Volume 46 December 1960 Number 12

Proceedings of the
National Academy of Sciences
of the United States of America

The Proceedings of the National Academy of Sciences

OFFICERS OF THE ACADEMY

DETLEV W. BRONK
President

FARRINGTON DANIELS
Vice President

H. P. ROBERTSON
Foreign Secretary

HUGH L. DRYDEN
Home Secretary

L. V. BERKNER
Treasurer

EDITORIAL BOARD OF THE PROCEEDINGS

SAUNDERS MAC LANE
Chairman

HUGH L. DRYDEN, *Home Secretary*
L. V. BERKNER, *Treasurer*
H. P. ROBERTSON, *Foreign Secretary*
EDWIN B. WILSON, *Managing Editor*

GREGORY BREIT	ERNST CLOOS	H. W. MAGOUN
HARRISON BROWN	THOMAS FRANCIS, JR.	HARLOW SHAPLEY
S. CHANDRASEKHAR	C. H. GRAHAM	PAUL WEISS

Subscription price is $12.50 for 1 year, $24.00 for 2 years, and $34.50 for 3 years; the price of a single issue is $1.50. Make all remittances payable to the National Academy of Sciences in U.S. currency or its equivalent.

Editorial correspondence should be addressed to the PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, 2101 Constitution Avenue, Washington 25, D. C.

Business correspondence should be addressed to the Printing and Publishing Office, National Academy of Sciences, 2101 Constitution Ave., Washington 25, D. C.

Subscribers are requested to notify the Printing and Publishing Office of the Academy and their local postmaster immediately of change of address. Notices to the Academy should provide both the old and the new address.

Microfilms of complete volumes of this journal are available to regular subscribers only and may be obtained at the end of the volume year from University Microfilms, 313 N. First Street, Ann Arbor, Michigan.

Second-class postage paid at Easton, Pennsylvania. This publication is authorized to be mailed at the special rates of postage prescribed by Section 132.122.

PRINTED IN THE U. S. A.

THE PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES
is published monthly by
THE NATIONAL ACADEMY OF SCIENCES

Proceedings of the
NATIONAL ACADEMY OF SCIENCES

Volume 46 · Number 12 · December 15, 1960

*THE LOSS OF PHENOTYPIC TRAITS BY DIFFERENTIATED CELLS IN VITRO, I. DEDIFFERENTIATION OF CARTILAGE CELLS**

BY H. HOLTZER,† J. ABBOTT, J. LASH,‡ AND S. HOLTZER

DEPARTMENT OF ANATOMY, SCHOOL OF MEDICINE, UNIVERSITY OF PENNSYLVANIA

Communicated by David R. Goddard and read before the Academy, November 16, 1960

The experiments to be described were designed to approach the following type of question: Will the differentiated state of a tissue cell survive multiple divisions *in vitro?* Do the cultured progeny of differentiated cells inherit the cellular mechanisms determining the unique somatic traits of their parental cells in a manner analogous to the way in which (1) Paramecia transmit Kappa particles to their daughter cells (Sonneborn,[33, 34] Beale,[2] Preer[31]), (2) bacteria inherit genes for constitutive or inducible enzymes in the absence of substrate (Novick and Weiner,[28] Spiegelman[35]) or (3) temperate phage particles give rise to lysogenic progeny (Jacob and Wollman[20])? Information of this kind is essential if the roles of the nucleus (i.e., genes) and the cytoplasm in cell differentiation are to be defined.

An unambiguous approach to these problems requires that the following five conditions be fulfilled: (1) The initial population of differentiated cells must be *homogeneous.* (2) The cells must divide many times during the experiment. (3) The cells must not back-differentiate to the parental type immediately after each division. (4) The initial population must consist of differentiated cells and not dividing precursor cells whose progeny differentiate and thereafter cease dividing (e.g., presumptive muscle cells, presumptive blood cells, presumptive pigment cells, etc.). (5) The somatic traits used as markers must be specific to the parent cell and readily characterized.

Given this list of conditions, it is clear that many kinds of somatic cells are excluded as test material. For example, both nerve cells and keratinizing skin cells do not divide. Liver and kidney cells offer difficulties of another kind; since it is difficult to secure a homogeneous population of either type of epithelial cell, it is possible that in long-term cultures the epithelial cells are selected against and the surviving population is derived from one of the "contaminating" cell types.

Cartilage cells from vertebrae of 10-day chick embryos, on the other hand, satisfy the five conditions outlined. Pieces of cartilage may be obtained which consist only of chondrocytes and a few chondroblasts.[15, 18] Chondrocytes retain their capacity to divide when liberated from their surrounding matrix, and they may be cultured under conditions which preclude their differentiation back to cartilage between cell divisions. Lastly, the metachromatic reaction of the mucopoly-

saccharide matrix with thionin and the incorporation of radioactive S^{35} into chondroitin sulfate allow identification of terminally differentiated cartilage cells.

Materials and Methods.—Cartilaginous vertebrae from 10-day chick embryos were stripped of muscle, connective tissue, and perichondrium. Microscopic examination of cartilage so prepared reveals few cells (considerably less than 1%) not embedded in a metachromatic matrix or surrounded by metachromatic capsules. The cartilage was cut into small pieces ($\sim$1 mm^3) and incubated in a 1 per cent trypsin (Difco 250) solution (Ca- and Mg-free Simms') for 2.5–3 hours.[26] The small pieces of cartilage were then transferred to a centrifuge tube and drained of excess trypsin. Two to three ml. of nutrient medium A (3 Simms' solution, 2 bovine serum ultrafiltrate, 1 embryo extract) were added and the cartilage cells liberated from the softened mucopolysaccharide matrix by flushing through small-bore pipettes. Insoluble fibrous material was removed by filtering through a Swinney filter in which a double thickness of lens paper replaced the millipore filter (Millipore Co.). The cells passing through the lens paper were spun down and the medium removed. The resultant pellet was resuspended in medium to a concentration of 7–10 $\times$ 10^4 cells/ml. One half ml aliquots of this suspension were pipetted into 8 to 10 Carrel flasks each of which had been coated with a plasma clot. The volume of the suspension *not* used to seed Carrel flasks was so adjusted that after centrifugation a pellet was obtained containing 5 $\times$ 10^6 cells. This pellet, consisting of freshly liberated chondrocytes, was organ-cultured on nutrient agar (1.2% agar in nutrient medium B). Nutrient medium B consisted of 2 parts Simms' solution, 2 parts horse serum, 1 part embryo extract.

The cells in the Carrel flasks were fed every 3rd day and subcultured anytime between the 2nd and 7th days. At this time, the cells, even though they had increased 2 to 7 times in number, presented a stellate, fibroblast-like appearance. The progeny of the liberated chondrocytes, grown in Carrel flasks, were resuspended, counted, and handled as the freshly liberated chondrocytes except that a 0.5 per cent trypsin solution was used for only 5 minutes and the suspension was not filtered through lens paper.

The organ-cultured pellets on nutrient agar, whether prepared from freshly liberated chondrocytes or from the resuspended progeny of the chondrocytes grown in Carrel flasks, were fed daily with nutrient medium B. After 10 days the organ-cultured pellets were fixed, stained, and examined for the presence of cartilage. Pellets from cells grown in Carrel flasks were also (1) grafted to the chorio-allantoic membrane of 9-day chick embryos or into the coeloem of 4-day chick embryos and (2) cultured by the ascending culture technique of Gaillard.[12]

To summarize: Freshly liberated chondrocytes were either immediately organ-cultured for 10 days or reared in Carrel flasks for varying periods. From time to time some of the progeny of the liberated chondrocytes were removed from the Carrel flasks, suspended, packed, and organ-cultured as pellets to see whether they still would form cartilage.

Thus far, 8 strains of chondrocytes have been established, and some strains maintained for as long as 60 days.

Embryonic cells are injured when dissociated by trypsin.[14] To understand better the growth curves of cells in the Carrel flasks, it proved desirable to determine the condition of the cells after trypsinization. For this purpose, entry into

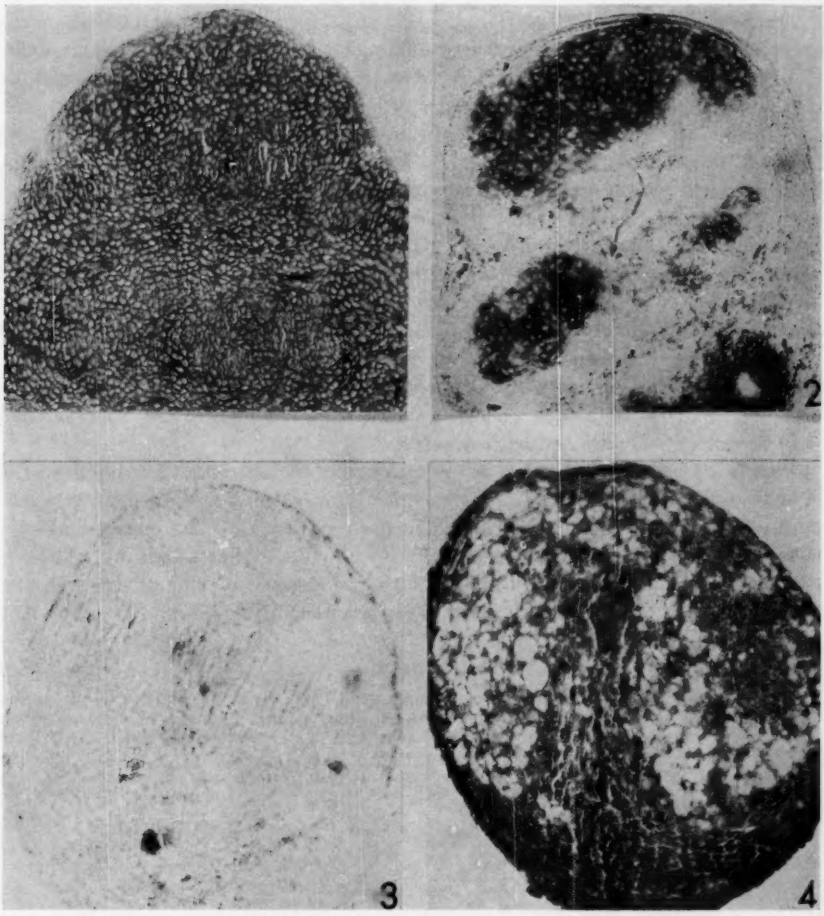


FIG. 1.—A pellet obtained from freshly liberated chondrocytes organ-cultured for 10 days, sectioned, and stained with thionin. It is estimated that over 98 per cent of the cells in the pellet are chondrocytes. Capsules and matrix stain metachromatically. (×450)

FIG. 2.—A pellet obtained from the progeny of chondrocytes grown in Carrel flasks for 4 days, organ-cultured for 10 days, sectioned, and stained with thionin. Between 40 per cent and 60 per cent of the cells in the pellet are chondrocytes with metachromatically staining capsules and matrix. The remaining cells are not stained or contain small intracellular metachromatic granules. (×450)

FIG. 3.—A pellet obtained from the progeny of chondrocytes grown in Carrel flasks for 15 days, organ-cultured for 10 days, sectioned and stained with thionin. There are no recognizable chondrocytes in the pellet. Scattered cells contain small intracellular metachromatic granules. (×450)

FIG. 4.—A companion pellet to that illustrated in Figure 3, but stained with hematoxylin and eosin. There are no recognizable chondrocytes or chondroblasts in the pellet. Many cells appear to be fat cells, and the large vacuoles are fixed and blackened by osmic acid. (×450)

injured cells of fluorescein-labeled rabbit globulin was used. The procedures for preparing and using labeled proteins for detecting damaged cells has been described by Holtzer and Holtzer.[17]

To follow histologically the chondrogenic activity of freshly liberated chondrocytes and their cultured progeny, three staining techniques were used: hematoxylin and eosin, Alcian blue, and thionin.[24] The term chondrocyte will be reserved for cells which exhibit a metachromatic extra cellular matrix with thionin.

Mature[4, 7] and embryonic[23, 29] chondrocytes actively incorporate inorganic sulfate, and the amount of ester-bound sulfate is an index of the amount of chondroitin sulfate synthesized by chondrocytes. After 6 days of organ culture on nutrient agar, the pellets were transferred to a piece of lens paper floating on 1.0 ml of nutrient medium B in a watch glass. One tenth of a ml of balanced salt containing 5.0 μc of $Na_2S^{35}O_4$ was added to each watch glass and the pellets incubated for an additional 3 days. The procedures used to extract and to identify the chondroitin sulfate synthesized in the tissues have been described in detail.[22] To facilitate comparisons between experiments, the radioactivity of the chondroitin sulfate was expressed as counts/5 $\times$ 10^6 cells, the number of cells in each organ-cultured pellet. Each pellet assayed for ester bound sulfate was matched with a control pellet stained with thionin.

Results.—Chondrogenic activity of freshly liberated chondrocytes: Freshly liberated chondrocytes from all 8 "strains" spun down into pellets and immediately organ-cultured invariably form a typical hyalin matrix (Fig. 1). The matrix and capsules are metachromatic after staining with thionin. Microscopically, the cells are typical chondrocytes. Estimates of the percentage of chondrocytes to the total population of cells in each pellet is of the order of 98% (Table 1). The fact

TABLE 1

THE CHONDROGENIC ACTIVITY OF FRESHLY LIBERATED CHONDROCYTES ORGAN-CULTURED FOR 10 DAYS CONTRASTED WITH THE CHONDROGENIC ACTIVITY OF THE PROGENY OF CHONDROCYTES GROWN FOR VARYING PERIODS IN CARREL FLASKS AND THEN ORGAN-CULTURED FOR 10 DAYS

Days in Carrel flasks	No. of pellets examined	Per cent chondrocytes in organ-cultured pellets	Per cent chondrocytes in chorion or coeloemic grafts
0	20	98	..
1.5	8	98	..
2	8	98	..
3	12	98	..
4	10	40–60	..
5	9	40–60	..
8	2	20–40	..
9	2	0	..
11	4	0	..
13	5	...	0
14	4	0	..
21	8	...	0
26	2	0	..
28	12	...	0
33	8	0	..
46	3	0	..
49	5	...	0

The figures of per cent chondrocytes in the individual pellets are visual approximations based on thionin-stained sections inspected under the microscope. The data in this table are derived from 8 different "strains" of chondrocytes isolated over a period of 8 months.

that almost all of the cells in the organ-cultured pellets are chondrocytes indicates that the initial population of cells is reasonably homogeneous.

The size of the pellets organ-cultured is not critical to chondrogenesis. Often pellets of freshly liberated chondrocytes fragment into smaller groups when placed

on agar. Aggregates of 20–30 cells behave as do collections of many thousands of cells.

Growth of the progeny of chondrocytes: Figure 5 illustrates the growth curves of 3 "strains" of cells derived from embryonic chondrocytes and grown in Carrel flasks. The variability in the over-all growth rates in the different "strains" is probably due to the trauma incident to trypsinizing the cells. That many freshly liberated cartilage cells and suspended cells from the Carrel flasks are injured by the cell

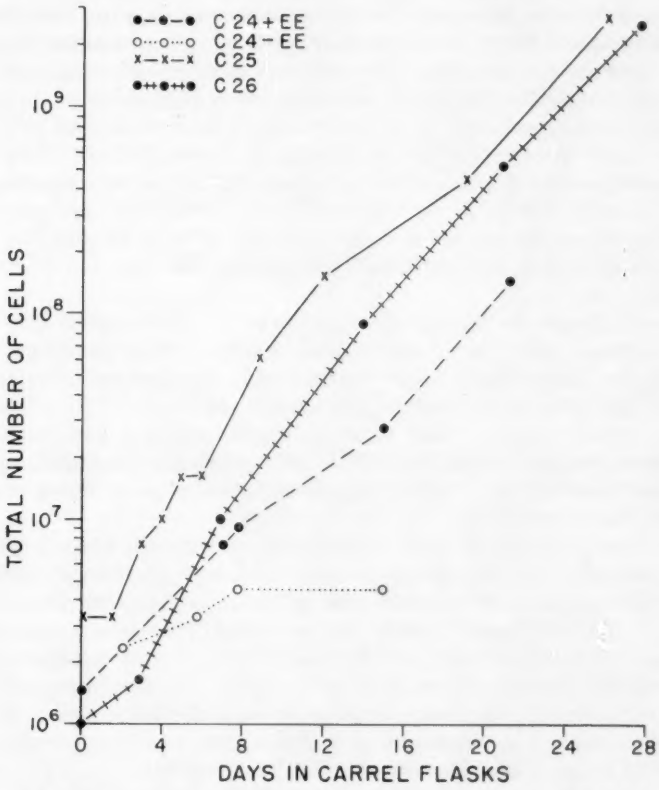


FIG. 5.—Growth curves of 3 "strains" of cells reared in Carrel flasks. See text for further details.

dissociation procedures is demonstrated by their uptake of fluorescein labeled rabbit globulin. Incubating the suspended cells for one hour in labeled globulin results in a diffuse fluorescence in the nucleus and cytoplasm of many of the cells. This reaction is characteristic for injured cells.[17] After trypsinization, the number of fluorescing cells varies from run to run: in some approximately 5%, in others as high as 20% of the total population take up the labeled protein. Chondrocytes in normal cartilage and cultured cells in Carrel flasks before trypsinizing do not take up the labeled protein.

The calculated generation time for the 8 "strains" used in these experiments is 34 hours. Owing, however, to the variable number of cells killed each time the cells are suspended the actual generation time must be considerably less. The growth curves make it improbable that the multiplying cells in the Carrel flasks are restricted to a small, actively dividing stem population.

Chondrogenic activity of the progeny of chondrocytes: Cells derived from chondrocytes grown in Carrel flasks lose their capacity to differentiate into cartilage when subsequently organ-cultured (Table 1 and Figs. 2, 3, and 4). Microscopic inspection of pellets consisting of cells reared 10 or more days in Carrel flasks and then organ-cultured fails to reveal the close packing of the rounded cells typical of chondroblasts or chondrocytes. The cells are healthy and occasional mitotic figures are found. The texture of the pellets varies from specimen to specimen and within a single specimen. In some regions the cells are tightly packed, whereas in other regions the ratio of cellular to extracellular material is low. When stained with thionin, scattered cells exhibit prominent metachromatic granules *within* individual cells. There is no extracellular metachromatic material. Many cells contain large vacuoles and give the impression of being fat cells with nucleus and cytoplasm peripherally displaced. The vacuoles are fixed and blackened by osmic acid.

There are changes in the aggregative behavior of cells reared in Carrel flasks which accompany their loss of chondrogenic activity. When placed on top of a *plasma clot*, a concentrated suspension of freshly liberated chondrocytes spontaneously aggregates into a nodular sheet within 24 hours. This nodular sheet does not spread radially. Most of these cells differentiate into chondrocytes. After several passages through Carrel flasks, the cells form a thin, rapidly spreading sheet when placed on top of a clot: they neither spontaneously aggregate nor differentiate into chondrocytes.

Effect of depressing growth rate in Carrel flasks: Cell division and cell differentiation are thought to be antagonistic processes and there are reports[6, 9] that when the division rate of dedifferentiated cells in culture is depressed, the cells redifferentiate. To test this, cells, after 15 days of rapid growth in normal medium, were reared for 6 days in Carrel flasks in the absence of embryo extract. Under these conditions, the cells did not increase in number, in contrast to control cells grown in the presence of embryo extract, which multiplied 6 times. The cells whose division rate was suppressed were organ-cultured. Chondrocytes did not differentiate in any of the nine pellets formed from these cells.

Behavior of cells in ascending cultures: Gaillard[12] reported that cultured embryonic chondroblasts reared in press juices from successively older embryos differentiate into mature chondrocytes and even form osteocytes. Cells after 4 passages through Carrel flasks were cultured as pellets in the press juices of 15-day, 18-day, and 20-day chick embryos. Eight pellets so treated did not differentiate into chondrocytes.

Behavior of cells in confronted cultures: Embryonic cartilage cells differentiate in association with striated muscle. Often, in mature animals, ectopic cartilage forms in muscle tissue.[21, 25] It was thought that muscle cells might provide an environment which would permit dedifferentiated cartilage cells to express latent chondrogenic capacities. Therefore pieces of 18-day chick muscle (1 mm^3 to 2

mm^3 in size) were excised and cultured with pellets from cells which had passed through five subcultures in Carrel flasks. Chondrocytes did not appear in 11 pellets grown under these conditions.

Behavior of cells in in vivo grafts: Culture conditions are not optimal for cell differentiation. For example, embryonic somites which fail to differentiate into cartilage *in vitro* differentiate into typical chondrocytes when grafted to the chorio-allantoic membrane.[1] Accordingly, the pellets formed from cells, passed through Carrel flasks 4 to 8 times, were grafted either to the chorio-allantoic membrane of 9-day embryos or to the coeloem of 4-day embryos. Neither the 18 grafts to the chorion nor the 12 grafts to the coeloem yielded chondrocytes.

Incorporation of radioactive sulfate: The quantitative experiments on the uptake of $S^{35}O_4$ parallel the results obtained on sectioned material stained with thionin.

TABLE 2

THE AMOUNT OF S^{35} BOUND BY PELLETS OF EQUAL CELL NUMBER GROWN ON NUTRIENT AGAR AFTER VARYING LENGTHS OF TIME IN CARREL FLASKS

Days in Carrel flasks	Total counts per minute per 5×10^6 cells grown in organ-culture	Per cent chondrocytes in organ-cultures
0	505,233	98
5	265,851	40–60
11	2,144	0
21	1,806	0
26	2,205	0

All the cells in this table were derived from the same "strain," were exposed to the same amount of $S^{35}O_4$, and were extracted in the same way. The estimate of per cent chondrocytes in the pellets is only approximate. Each measurement is based on the mean value of 5 cultures.

Table 2 and Figure 6, part A, show that the incorporation of S^{35} into bound sulfate by cells in pellets in organ culture varies inversely with the time grown in Carrel flasks. Table 2 correlates the histological condition of the organ-cultured pellets with the amount of ester-bound isotope.

Part B of Figure 6 suggests that the loss of chondrogenic activity of cells grown in Carrel flasks is a function of at least two variables—the number of divisions the cells have experienced and the length of time cultured. Freshly liberated chondrocytes were cultured for 3 days in Carrel flasks and then divided into two populations. One population was grown in flasks in the presence of embryo extract (+EE), the other in flasks in which the embryo extract was omitted (−EE). The growth curves of these two populations is indicated in Figure 5 (C24 + EE and C24 − EE). The loss of chondrogenic activity of cells grown in the absence of embryo extract for the first 8 days is considerably less than cells grown in the presence of embryo extract. During this period, the −EE cells have doubled in number, whereas the +EE cells have increased over fourfold. Between the 8th and 15th day in the flasks, the −EE cells have not increased in total number, while the +EE cells have multiplied 3 times. Nevertheless, during the period from the 8th to the 15th day, both groups of cells lose their capacity to differentiate into chondrocytes.

Discussion.—"Dedifferentiation" is used in this paper as a convenient word to describe cells derived from mature chondrocytes which have lost the ability to form recognizable chondrocytes and to synthesize chondroitin sulfate. It is not meant to imply a reversion to an "embryonic state." Failure of these cells to

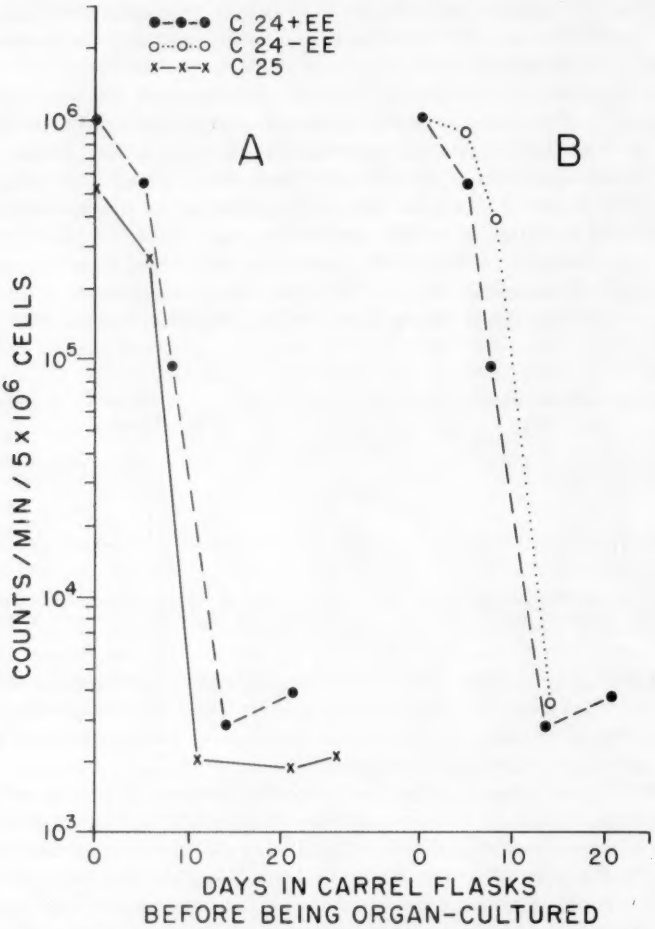


FIG. 6.—*Part A:* The amount of S^{35} bound by two "strains" of chondrocytes and their progeny grown as pellets in organ culture. Pellets with counts below 10,000 did not form microscopically recognizable cartilage and they failed to yield detectable quantities of glucuronic acid or galactosamine.

Part B: Cells of "strain" C24 were divided into two groups and grown in Carrel flasks: C24 +EE were grown in the presence of embryo extract; C24 − EE were grown in the absence of embryo extract. The reduced growth rate of the cells grown in the absence of embryo extract is shown in Figure 5. Note that the pellets formed from cells which underwent fewer divisions for the first eight days in Carrel flasks, when organ-cultured, bound greater amounts of S^{35} than the more rapidly dividing cells. After approximately 10 days in Carrel flasks, both strains lose the capacity to synthesize insoluble polysaccharide when organ-cultured.

form chondrocytes is unlikely to be due to failure to achieve a critical mass or to general environmental deficiencies. Presumptive embryonic and regenerating cartilage cells differentiate under a variety of *in vivo* and *in vitro* conditions too restrictive for other types of cells (see examples in refs. 13, 15, 16). Rather, it is

assumed that rapidly dividing chondrocytes lose one or more factors indispensable for the formation of chondroitin sulfate. Whether this loss is cytoplasmic or nuclear, whether it is due to dilution of an enzyme or a critical part of the cell surface, or whether it is reversible or irreversible is unknown. From the work of Hsu,[19] it is unlikely that ploidy is implicated, for chromosomal aberrations in cultured cells appear only in cells grown for longer periods of time.

Perske, Parks, and Walker[30] report a comparable case of dedifferentiation where liver cells in culture lose several enzymes. Likewise, Eagle's report[8] on the similar metabolic requirements of a variety of cells in culture can be interpreted as the dedifferentiation of the progeny of specialized cells. After embryonic muscle or notochord is grown in Carrel flasks for several generations, cells are recovered which do not synthesize myosin or form vacuolated cells.[15] On the other hand, Schindler, Day, and Fisher[32] report that mast tumor cells in culture continue to produce 5-hydroxytryptamine, thus demonstrating the preservation of specialized metabolic pathways in at least one type of malignant cell (see also Waltz *et al.*[37] on the formation of progesterone by hydatiform mole in culture).

Clearly, the old problem of "dedifferentiation vs. modulation" of cells in tissue culture is by no means settled (see reviews by Bloom,[3] Weiss,[38] Ephrussi,[10,11] and Trinkaus[36]). That chondrocytes and probably other somatic cells do dedifferentiate is, in our opinion, no longer debatable. But the interesting problems remain: (1) Does a given chondrocyte dedifferentiate gradually or abruptly? (2) How dependent upon mitotic divisions is the process of dedifferentiation? (3) Do dedifferentiated chondrocytes continue to synthesize collagen or the protein associated with chondroitin? (4) Can dedifferentiated chondrocytes differentiate into other types of cells or can they only differentiate back into chondrocytes?

In spite of many theoretical discussions, virtually nothing is known of the respective roles of the nucleus and cytoplasm in differentiating cells.[5, 27] Nevertheless, the results of these experiments on dedifferentiation invite one speculative comment: If the mitotic divisions of the cultured cells transmit the functioning genes of the original chondrocytes, then it must be a cytoplasmic difference which distinguishes the dedifferentiated cells from their parental chondrocytes. Whatever the role of genes in controlling enzymes responsible for the synthesis of chondrotin sulfate, such activity may be inhibited by an altered cytoplasm. Whether such inhibition is mediated by the activation of "regulatory" genes or is confined to cytoplasmic mechanisms is unknown.

Summary.—Chondrocytes liberated from embryonic cartilage by means of trypsin and immediately organ-cultured continue to synthesize chondroitin sulfate and incorporate inorganic S^{35} into ester-bound sulfate. After being cultured under conditions that suppress differentiation but encourage cell multiplication, these cells, when organ-cultured, (1) fail to differentiate into recognizable chondrocytes, (2) fail to synthesize chondroitin sulfate, and (3) fail to incorporate S^{35} into ester-bound sulfate. Dedifferentiated cartilage cells may differentiate into fat cells.

* This investigation was supported, in part, by Research Grants B-493 and B-1629 from the National Institute of Neurological Diseases and Blindness of the National Institutes of Health, U.S. Public Health Service.

† Scholar in Cancer Research of the American Cancer Society, Inc.

‡ Helen Hay Whitney Foundation Research Fellow.

[1] Avery, G., M. Chow, and H. Holtzer, *J. Exp. Zool.*, **132,** 409 (1958).

[2] Beale, G., *The Genetics of Paramecium aurelia* (Cambridge University Press, 1954).

[3] Bloom, W., *Physiol. Rev.*, **17,** 589 (1937).

[4] Bostrom, H., *J. Biol. Chem.*, **196,** 477 (1952).

[5] Briggs, R., and T. King, in *The Cell*, ed. J. Brachet and A. Mirsky (New York: Academic Press, 1959), pp. 537–611.

[6] Doljansky, L., *C. V. Soc. Biol., Paris*, **105,** 343 (1930).

[7] Dziewiatsowski, D., *Int. Rev. Cytology*, **7,** 59 (1958).

[8] Eagle, H., in *Harvey Lectures*, **54,** 156 (1960).

[9] Ebling, A. H., and A. Fischer, *J. Exp. Med.*, **36,** 285 (1922).

[10] Ephrussi, B., in *Enzymes: Units of Biological Structure and Function*, ed. O. H. Gaebler (New York: Academic Press, 1956), pp. 29–40.

[11] Ephrussi, B., *J. Cell and Comp. Physio.*, Suppl. I, **52,** 35–55.

[12] Gaillard, P., *Actualitiés Scientifiques et Industrielles* (Paris: Hermann et Cie, 1942), p. 923.

[13] Holtzer, H., in *Regeneration in Vertebrates*, ed. C. Thornton (University of Chicago Press, 1958).

[14] Holtzer, H., *Exp. Cell Res.*, Suppl. 7, 234 (1959).

[15] Holtzer, H., in *19th Growth Symposium* (in press), ed. D. Rudnick (New York: Ronald Press, 1960).

[16] Holtzer, H., and S. Detwiler, *J. Exp. Zool.*, **123,** 335 (1953).

[17] Holtzer, H., and S. Holtzer, *Compt. Ren. Trav. (Carlsberg), ser. chim.*, **31,** 373 (1960).

[18] Holtzer, H., J. Abbott, and J. Lash, *Anat. Rec.*, **131,** (1958).

[19] Hsu, T., in *18th Growth Symposium*, ed. D. Rudnick (New York: Ronald Press, 1959).

[20] Jacob, F., and E. L. Wollman, in *The Chemical Basis of Heredity*, ed. W. D. McElroy and B. Glass (Baltimore: The Johns Hopkins Press, 1958).

[21] La Croix, P., *The Organization of Bones* (Philadelphia: Blakiston Press, 1950).

[22] Lash, J., H. Holtzer, and M. Whitehouse, *Dev. Biol.*, **2,** 76 (1960).

[23] Layton, L., *Proc. Soc. Exp. Biol. and Med.*, **76,** 596 (1951).

[24] Lillie, R. D., *Histopathologic Technic and Practical Histochemistry* (New York: McGraw-Hill Book Company, 1954).

[25] McLean, F. C., and M. R. Urist, *Bone: An Introduction to the Physiology of Skeletal Tissue* (University of Chicago Press, 1955).

[26] Moscona, A., *Exp. Cell Res.*, **3,** 535 (1952).

[27] Nanney, D. L., in *The Chemical Basis of Heredity*, ed. W. D. McElroy and B. Glass (Baltimore: The Johns Hopkins Press, 1958).

[28] Novick, A., and M. Weiner, these PROCEEDINGS, **43,** 553 (1957).

[29] Pelc, S., and A. Glücksmann, *Exp. Cell Res.*, **8,** 336 (1955).

[30] Perske, W. F., R. E. Parks, Jr., and D. Walker, *Science*, **125,** 1290 (1957).

[31] Preer, J., in *Developmental Cytology*, ed. D. Rudnick (New York: Ronald Press, 1959).

[32] Schindler, R., M. Day, and G. Fisher, *Cancer Res.*, **19,** 47 (1959).

[33] Sonneborn, T., *Harvey Lectures*, **44,** 145 (1950).

[34] Sonneborn, T., these PROCEEDINGS, **46,** 149 (1960).

[35] Spiegelman, S., in *The Chemical Basis of Heredity*, ed. W. D. McElroy and B. Glass (Baltimore: The Johns Hopkins Press, 1958).

[36] Trinkaus, J. P., *Am. Naturalist*, **90,** 273 (1956).

[37] Waltz, H. K., W. W. Tullner, V. J. Evane, R. Hertz, and W. R. Earle, *J. Natl. Cancer Inst.* **14,** 1173 (1954).

[38] Weiss, P., *Quart. Rev. Biol.*, **25,** 177 (1950).

*SOME OBSERVATIONS ON THE SYNTHESIS AND FUNCTION OF THE PHOTOSYNTHETIC APPARATUS IN RHODOSPIRILLUM RUBRUM**

By Germaine Cohen-Bazire and Riyo Kunisawa

DEPARTMENT OF BACTERIOLOGY, UNIVERSITY OF CALIFORNIA, BERKELEY

Communicated by I. Michael Lerner, September 26, 1960

The chlorophyll content of nonsulfur purple bacteria varies greatly in response to environmental factors, of which the most important are light intensity and oxygen.[1] In cells growing anaerobically in the light (i.e., under conditions of obligatory photosynthesis), the differential rate of chlorophyll synthesis is an inverse function of light intensity. Many nonsulfur purple bacteria can also grow aerobically, at the expense of respiratory metabolism. Oxygen inhibits chlorophyll synthesis, and accordingly cells of such purple bacteria grown under stringent aerobic conditions, whether in the dark or in the light, are devoid of chlorophyll. Chlorophyll synthesis can, however, take place even in the dark at very low oxygen tensions. Consequently cultures grown with a restricted oxygen supply contain considerable amounts of pigment. The observations concerning environmentally conditioned changes in the chlorophyll content of purple bacteria have so far all been made on intact cells.

In bacteria, the photosynthetic pigment system can be isolated from disrupted cells in association with macromolecular particles known as chromatophores.[2] These particles are chemically complex, and contain as major constituents both protein and phospholipid, in addition to the photosynthetic pigments.[3, 4] They are the site of the primary photochemical reaction of bacterial photosynthesis, cyclic photophosphorylation.[5–7]

It is not clear whether gross changes in cellular chlorophyll content simply reflect fluctuations in the rate of chlorophyll synthesis and incorporation into an otherwise constant chromatophore structure, or whether they are one manifestation of much more complex, environmentally induced, changes in the biosynthesis, composition, and function of the whole chromatophore system.

This question can be answered only by the isolation of purified chromatophores from cells with different chlorophyll contents, followed by the systematic comparison of the various properties of such chromatophores. The present paper describes the influence of environmental factors that affect the chlorophyll content of whole cells on the specific chlorophyll content, the photophosphorylative capacity, and the succinic dehydrogenase activity of isolated chromatophores. The two enzymatic activities mentioned have both been shown to be associated with the chromatophores of purple bacteria.[6, 8] The former activity is clearly dependent on the presence of a photochemically functional pigment system, whereas the latter is not. Comparative measurements of these two activities could therefore be expected to reveal any differences between photochemically linked and strictly "dark" enzymatic functions which might occur in chromatophores prepared from cells grown under different conditions.

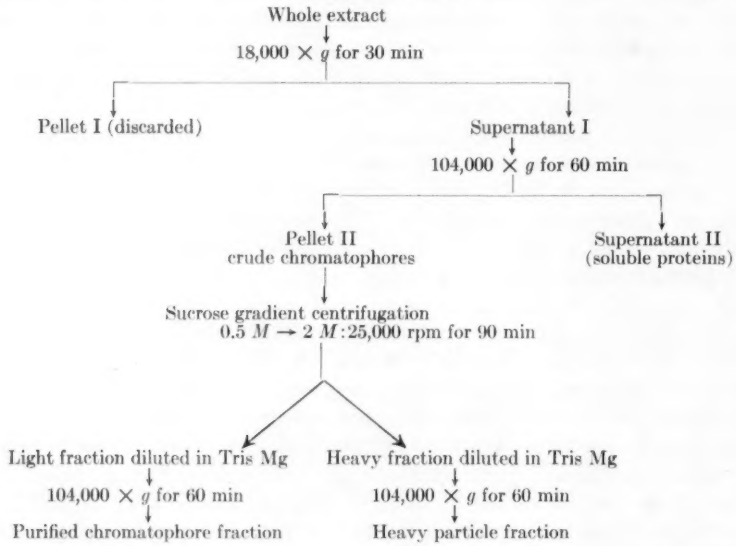
Materials and Methods.—Bacterial cultivation: Rhodospirillum rubrum, strain 1.1.1., from the collection of Professor C. B. van Niel was used in all experiments. Photosynthetic cultures were grown in the modified medium of Hutner,[1] with

malate as carbon source. The composition of the medium and the technical details of cultivation have already been described.[1, 9] Aerobic depigmented cultures were grown in the complete absence of light with mechanical agitation in Fernbach flasks (2.5-liter capacity), containing 500 ml of Hutner medium with succinate as carbon source. The inoculum consisted of depigmented cells, grown in the dark with strong aeration. Cultures were harvested during exponential growth, at a population density of less than 2×10^8 bacteria per ml. All cultures were grown at 30°C.

Measurements on cell suspensions and extracts: Cell mass was determined by measuring the optical density of cultures at 680 mμ in a Beckman DU spectrophotometer. The bacteriochlorophyll content of cell suspensions or cellular fractions was determined spectrophotometrically.[1] The Folin-Lowry method[10] was

TABLE 1

PROCEDURE USED FOR THE ISOLATION OF PURIFIED CHROMATOPHORES FROM EXTRACTS OF *Rhodospirillum rubrum*


Whole extract
18,000 × g for 30 min
Pellet I (discarded)
Supernatant I
104,000 × g for 60 min
Pellet II
crude chromatophores
Supernatant II
(soluble proteins)
Sucrose gradient centrifugation
0.5 M → 2 M:25,000 rpm for 90 min
Light fraction diluted in Tris Mg
Heavy fraction diluted in Tris Mg
104,000 × g for 60 min
104,000 × g for 60 min
Purified chromatophore fraction
Heavy particle fraction

used to measure the protein content of cell extracts and of cellular fractions. At the dilutions used for protein determination, the photosynthetic pigments, if present, do not interfere with the analysis. The protein content of intact cells of *R. rubrum* cannot be satisfactorily determined by either the Biuret or the Folin-Lowry method. In order to use the Biuret method, photosynthetic pigments must first be extracted with acetone-methanol, which also removes alcohol-soluble proteins. The Folin-Lowry method could in principle be used after alkaline digestion; however, the cells of *R. rubrum* are not digested completely in *N* NaOH, and the values for protein are low and not reproducible. When it was necessary to estimate the protein content of a cell suspension, the cells were first broken, and the estimation was made on the whole extract; this gave consistent and reproducible results.

Preparation and purification of bacterial chromatophores: Cells were harvested by centrifugation at 4°C, washed, and resuspended in chilled phosphate buffer (0.02 *M*, pH 7) containing 0.01 *M* $MgSO_4$. All subsequent operations were performed at 4°C. The concentrated suspension of cells in buffer was disintegrated in the French Pressure Cell (Aminco) under a pressure of 20,000 lb per square inch. The whole extract was then fractionated by the procedure outlined in Table 1. The first centrifugation at 18,000 × *g* for 30 min eliminated the whole cells and coarse debris and gave a transparent, pigmented supernatant which was subsequently centrifuged for one hour in a Spinco preparative centrifuge at 104,000 × *g*. The orange-pink supernatant liquid, containing the soluble proteins and large amounts of cytochrome c_2[11], was discarded. The pellet constituted the *crude chromatophore fraction*. The wall of the centrifuge tube as well as the pellet were rinsed with Tris-HCl buffer (0.33 *M*, pH 7.6) containing 0.01 *M* $MgSO_4$ (Tris-Mg buffer). The pellet of crude chromatophores was resuspended in a small volume of Tris-Mg buffer and the suspension homogenized with a hypodermic syringe. Part of the crude chromatophore suspension was kept for tests of enzymatic activity and analysis of pigment and protein content. The remainder was centrifuged through a linear sucrose density gradient in the swinging bucket rotor S W 25–1 of the preparative Spinco centrifuge, operated at 25,000 rpm for 90 min. The linear density gradient was established beforehand, in centrifuge tubes lightly coated with silicone grease. The mixing device of Bock and Ling[12] permitted the establishment of a linear gradient, using equal volumes of two sucrose solutions of different molarities.[13] We used routinely 11 ml of 2 *M* sucrose and 11 ml of 0.5 *M* sucrose to establish the gradient. The sucrose solutions were prepared in Tris-Mg buffer. Another linear sucrose gradient, using 2 ml of 0.4 *M* sucrose and 2 ml of the crude chromatophore suspension to be analyzed, was layered on top of the first gradient with a smaller mixing device. An equally satisfactory procedure is to layer with a slow flowing pipette on top of the first gradient a relatively small volume (1.5 ml) of a concentrated crude chromatophore suspension in Tris-Mg buffer or in 0.2 *M* sucrose.

After centrifugation at 25,000 rpm for 90 min, the gradient shows two main pigmented bands. The bottom one scatters light strongly and has a gelatinous consistency; it contains what we call the "heavy particle fraction." The upper band is pigmented and does not scatter light appreciably; we call the material in this band the "purified chromatophore fraction." The position of the middle of the purified chromatophore band (upper band) in the gradient corresponds to a concentration of sucrose of approximately 1.08 *M*. The lower pigmented band corresponds to a sucrose concentration varying between 1.77 and 1.8 *M*. It was not in general possible to collect the fractions directly by elution; most of the heavy fraction was first carefully removed with a thin Pasteur pipette without disturbing the gradient. The remaining fractions were collected by elution through a small hole punctured at the bottom of the tube. The fractions were diluted 3–5 fold with Tris-Mg buffer, and centrifuged for one hour at 104,000 × *g*. The colorless supernatant liquids were discarded, the sides of the tubes were rinsed, and the pellets were resuspended in a small volume of Tris-Mg buffer.

Enzymatic assays: Photophosphorylating activity[14, 15] was measured by determining the disappearance of inorganic phosphate after 30 min of illumination

at 30°C ± 1°C under anaerobic conditions. The light intensity was approximately 1,000 ft.-c. at the surface of the reaction vessels. The reaction mixture contained: 20 μmoles of potassium phosphate buffer (pH 7.6), 1 μmole of ADP, 50 μmoles of mannose, 1 mg. of yeast hexokinase, 0.2 μmole of sodium succinate, 20 μmoles of $MgCl_2$, and 7–10 μmoles of Tris-HCl buffer (pH 7.6). The material to be assayed contained 1–5 mg. of protein, and 10–100 μg of bacteriochlorophyll. The total volume varied between 1 and 1.2 ml. In some experiments, methylphenazonium methosulfate (PMS) was added to the reaction system at a concentration of 0.2 mg per ml. In such cases, a piece of transparent cellophane, absorbing light below 500 mμ, was placed against the bottom of the reaction vessels to prevent destruction of the dye by light.[15] The reaction was performed in rectangular Warburg vessels in the Bronwill Warburg apparatus model UVL. The illuminated surface of the flasks measured approximately 10 cm^2 and the reaction mixture accordingly had a depth of approximately 1 mm. Inorganic phosphate was measured by the method of Fiske and Subbarow[16] on an aliquot of the reaction mixture previously deproteinized by the addition of three volumes of 18 per cent trichloracetic acid. Two measures of specific photophosphorylative activity were used: micromoles of inorganic phosphate esterified per hour per milligram of chlorophyll; and micromoles of inorganic phosphate esterified per hour per milligram of protein. These values will be designed, respectively, as $Q_{Pi\ (chlorophyll)}$ and $Q_{Pi\ (protein)}$.

Succinic dehydrogenase activity was determined by manometric measurement of the oxygen uptake during 30 min at 30°C in the dark, in the presence of PMS.[17] The reaction mixture contained, in a final volume of 1.4 ml, 20 μmoles of sodium succinate, 100 μmoles of potassium phosphate (pH 7.4), 10 μmoles of $MgCl_2$, and 2 mg of PMS. The fractions assayed had a protein content of 0.5–1.5 mg. The reaction was started by tipping substrate and PMS from the side arm. The rate of oxygen uptake remained constant during the period of measurement. The very dilute preparations assayed consumed no oxygen in the absence of PMS, and the addition of KCN to block transport through the cytochrome chain could therefore be dispensed with. Phosphate was necessary for maximal activity. The specific activity of succinic dehydrogenase is expressed as μmoles of succinate oxidized per hour per mg protein, abbreviated as $Q_{succ.\ (protein)}$.

Purified pancreatic lipase was prepared from crude steapsin by the method of Glick and King.[18] Lipase digestion was carried out at 30°C for 2 hr, using 23 μg protein of purified lipase per ml of chromatophore suspension. The hexokinase and crude pancreatic lipase (steapsin) were products of the Nutritional Biochemicals Corporation.

Results.—Properties of the purified chromatophore fraction: Depending on the conditions of cultivation of the cells and the method of breakage employed, the purified chromatophore fraction represents from a third to a half of the chlorophyll-containing material recoverable from the sucrose gradient. Its specific chlorophyll content on a protein basis is always appreciably higher (by 10–30%) than that of the crude chromatophore preparation. The lower band, which we have termed the *heavy particle fraction*, always contains at least half and usually more of the chlorophyll-containing material recoverable from the gradient. Its specific chlorophyll content is in general slightly lower than that of the crude chromatophore preparation. The two enzymatic functions that we have examined—photophos-

phorylation and succinic dehydrogenase activity—occur in both purified chromatophores and heavy particles; specific activities are higher on a protein basis in the purified chromatophores than in crude chromatophores and heavy particles. Furthermore, the enrichment of the two functional properties in purified chromatophores parallels the enrichment of chlorophyll (Table 2). PMS increases photophosphorylative activity, but the relative increase is the same for each fraction.

TABLE 2

SPECIFIC ENZYMATIC ACTIVITIES OF CHROMATOPHORE FRACTIONS ISOLATED FROM PHOTOSYNTHETICALLY GROWN CELLS OF *Rhodospirillum rubrum*

	Cells grown at high light intensity (~2,000 ft.-c.)					
	Crude chromatophores		Purified chromatophores		Heavy fraction	
	I*	II*	I	II	I	II
Specific chlorophyll: μg per mg protein	15.4	16.9	20.8	23.2	14.3	18.2
Photophosphorylation:						
$Q_{Pi\ (chlorophyll)}$ No PMS	547	485	738	645	283	241
$Q_{Pi\ (chlorophyll)}$ PMS	..	888	1,110	1,100	..	512
$Q_{Pi\ (protein)}$ No PMS	8.62	8.2	15.3	15	4.06	4.4
$Q_{Pi\ (protein)}$ PMS	..	15	25	25.7	..	9.12
Succinic dehydrogenase: $Q_{succ.\ (protein)}$	8.25	..	13	...	8.2	..
	Cells grown at low light intensity (~200 ft.-c.)					
Specific chlorophyll: μg per mg protein	45.6	..	55.7	...	37.4	
Photophosphorylation:						
$Q_{Pi\ (chlorophyll)}$ No PMS	225	..	293	...	115	
$Q_{Pi\ (chlorophyll)}$ PMS	359	..	450	...	271	
$Q_{Pi\ (protein)}$ No PMS	10.4	..	16.3	...	4.32	
$Q_{Pi\ (protein)}$ PMS	16.5	..	25	...	10	
Succinic dehydrogenase: $Q_{succ.\ (protein)}$	7.4	..	12.6	...	7.3	

* I and * II represent two independent experiments.

Treatment with pancreatic lipase has a dramatic effect on the physical properties of the heavy particles. When a treated suspension of heavy particles is washed free of enzyme by centrifugation and then again subjected to a sucrose gradient centrifugation, all the chlorophyll-containing material can be recovered in particles that form a band in the gradient corresponding in position to that of purified chromatophores; however, the particles recoverable from the heavy fraction after lipase treatment have a higher specific chlorophyll content than the purified chromatophores isolated directly from the same crude chromatophore preparation. When the purified chromatophores are subjected to lipase treatment, their migration in the sucrose gradient does not change; however, their specific chlorophyll content increases substantially, to a value the same as that of the particles recoverable after lipase treatment of the heavy particle fraction. Data illustrating the effects of lipase treatment on purified chromatophores and on the heavy fraction prepared from cells grown at two different light intensities are shown in Table 3. It can be seen that the degree of chlorophyll enrichment achieved by lipase treatment of purified chromatophores is much greater for chromatophores prepared from cells grown at high light intensity, which have a low specific chlorophyll content, than

for the chromatophores prepared from cells grown at low light intensity. The particles obtained after lipase treatment retain the symmetrical infrared absorption band with a maximum at 885 mμ characteristic of the bacteriochlorophyll of *R. rubrum* in its native state; however, they are completely devoid of the capacity to photophosphorylate or to dehydrogenate succinate.

TABLE 3

EFFECT OF PANCREATIC LIPASE ON THE SPECIFIC CHLOROPHYLL CONTENT OF PURIFIED CHROMATOPHORES AND HEAVY PARTICULATE FRACTION OF *R. rubrum* GROWN PHOTOSYNTHETICALLY AT HIGH AND LOW LIGHT INTENSITY

	Specific chlorophyll: μg per mg protein	
	High light intensity (2,000 ft.-c.)	Low light intensity (200 ft.-c.)
Purified chromatophores	22.2	65.5
Heavy fraction	13	53
Lipase treated purified chromatophores	51	88.8
Lipase treated heavy fraction	54	87.5
Enrichment in specific chlorophyll after lipase treatment of purified chromatophores	2.3	1.35

In effect, therefore, the light fraction isolated by sucrose gradient centrifugation of crude chromatophore material is the most highly purified enzymatically functional chromatophore fraction that we have been able to isolate. A particulate fraction more highly purified in terms of pigment content is obtainable by lipase treatment; but such treatment abolishes enzymatic function. Since the primary aim of this work was to examine the relationship between the pigment content and the functional activity of the photosynthetic apparatus, the properties of the lipase-treated particles were not further investigated.

The relationship between the chlorophyll content of cells and chromatophores: Table 4 shows the specific chlorophyll content of crude bacterial extracts and purified

TABLE 4

SPECIFIC BACTERIOCHLOROPHYLL CONTENT OF WHOLE EXTRACTS AND PURIFIED CHROMATOPHORES PREPARED FROM *R. rubrum* GROWN PHOTOSYNTHETICALLY AT HIGH AND LOW LIGHT INTENSITY

	Specific bacteriochlorophyll content μg per mg protein		Percentage of total cell protein present in chromatophore material
	Crude extracts	Purified chromatophores	
Cells grown at high light intensity 2,000 ft.-c.	9.3	23.2	40
	10.2	24.9	41
	10.3	22.2	46.5
	11.7	24.4	48
Average value	10.4	23.7	45
Cells grown at low light intensity (200 ft.-c.)	26.7	49.5	54
	26.9	55.7	48
	35.8	65.5	55
	36.4	60.5	60
Average value	31.4	57.8	54

chromatophores prepared from four different batches of cells grown photosynthetically in malate medium under steady-state conditions of pigment synthesis at two different light intensities (*ca.* 200 and 2,000 ft.-c.). The specific chlorophyll content of crude extracts from cells grown at the lower light intensity is about three times as great as that of crude extracts from cells grown at the

higher light intensity. The purified chromatophores likewise show a very marked difference in specific chlorophyll content, paralleling that of the crude extracts. Hence it follows that the regulation of the chlorophyll content of whole cells in response to changes of light intensity is achieved primarily by a change in the chlorophyll content of the chromatophores, rather than by a change in the amount of chromatophore material per cell.

It is at present impossible to determine the chromatophore content of photosynthetic bacteria directly, since no method exists for the quantitative isolation of the photosynthetic apparatus in a physically homogeneous state. However, the fraction of cell protein represented by chromatophores can be derived from data on the specific chlorophyll content of whole extracts and of purified chromatophores. Calculated chromatophore contents, expressed in these terms, are shown in the last column of Table 4. The values suggest that the fraction of total cell protein represented by chromatophores is slightly greater in cells grown at low light intensity. The effect of light intensity on chromatophore content is, however, negligible in comparison with its effect on chlorophyll content.

The influence of chlorophyll content on the functional properties of chromatophores: Table 5 shows the specific activities of photophosphorylation and succinic dehydrogenase in purified chromatophores prepared from cells grown photosynthetically at different light intensities. The rate of photophosphorylation is presented both as a function of chlorophyll content—the measure of specific activity used by most workers[6]—and as a function of protein content. The latter values are relatively constant, and such variations as do occur are not systematically correlated with the light intensity at which the cells were grown. The former values vary almost sixfold, and are inversely related to the specific chlorophyll content of the chromatophores (Fig. 1). Since the rate of photophosphorylation was always measured at a saturating light intensity, it follows that this rate must have been limited not by the primary photochemical reaction (i.e., by the amount of chlorophyll present), but rather by one of the subsequent dark reactions. The specific activity of succinic dehydrogenase is remarkably constant, and does not differ significantly in chromatophores from cells grown at different light intensities (see also Table 2).

TABLE 5

ACTIVITIES OF PURIFIED CHROMATOPHORES EXTRACTED FROM *R. rubrum* GROWN PHOTOSYNTHETICALLY AT DIFFERENT LIGHT INTENSITIES

Approximate light intensity →	2,000 ft.-c.			1,000 ft.-c.	200 ft.-c.		100 ft.-c.
	I	II	III	I	I	II	I
Specific chlorophyll: (μg per mg protein)	20.8	23.2	24.9	43	55.7	60.5	77.6
Photophosphorylation:							
$Q_{Pi\ (chlorophyll)}$							
No PMS	738	645	645	334	293	179	130
PMS	1,110	1,100	..	..	450	..	..
$Q_{Pi\ (protein)}$							
No PMS	15.3	15	14.4	14.7	16.3	10.8	10
PMS	25	25.7	..	..	25	..	..
Succinic dehydrogenase:							
$Q_{succ.\ (protein)}$	13.2	12.4	13	..	12.6	13.6	..

The formation of chlorophyll and chromatophores in cells initially free of pigment: Since air completely suppresses chlorophyll synthesis by *R. rubrum*, cells that have been grown for many generations in the dark under strictly aerobic conditions are devoid of chlorophyll. At very low oxygen tensions, such cells can initiate chlorophyll synthesis even in the absence of light.[19] Under these circumstances, growth is exceedingly slow. Nevertheless, chlorophyll synthesis is rapid, and after a few hours of incubation under conditions of oxygen limitation the cells attain a chlorophyll content comparable with that of cells grown anaerobically in the light (i.e., under conditions of obligatory photosynthesis). Preliminary experiments showed that the chromatophores isolated from such "semi-aerobic" cells, grown in the complete absence of light, could perform photophosphorylation. A detailed study of the synthesis of chlorophyll and the development of photophosphorylative capacity during semi-aerobic growth of initially depigmented cells was therefore undertaken.

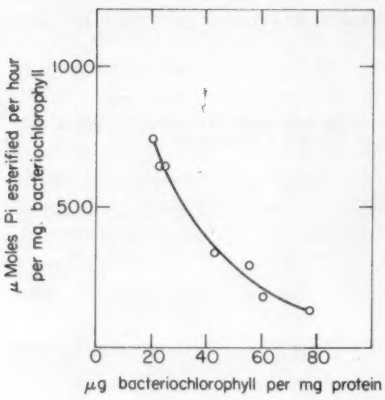


FIG. 1.

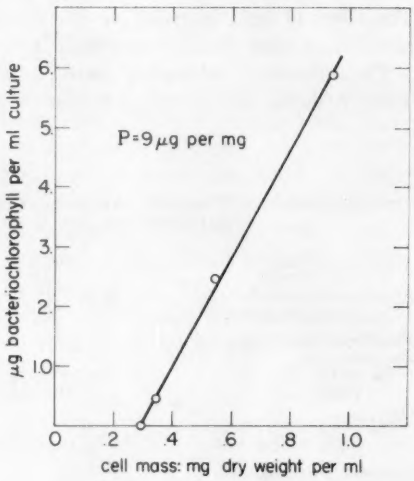


FIG. 2.

While still in the course of exponential growth under strictly aerobic conditions in the dark, several cultures of depigmented cells were pooled, and 750 ml of the pooled suspension was placed in each of several 1-liter Erlenmeyer flasks. These flasks were further incubated in the dark with gentle agitation. At appropriate intervals, the cells from a single flask were harvested for analysis. Oxygen is the limiting nutrient under these conditions, and the growth of the population is regulated by the rate of oxygen diffusion into the culture, and by the rate of its consumption. The kinetics of growth cannot, therefore, be precisely defined. Nevertheless, as shown in Figure 2, the increase in chlorophyll is strictly proportional to the increase in cell mass for several hours. In order to obtain a high differential rate of chlorophyll synthesis, the use of succinate as a carbon source is essential. Malate, although equivalent to succinate as a substrate for growth, supports little or no chlorophyll synthesis under semiaerobic conditions. With succinate as a carbon source, the differential rate of chlorophyll synthesis, P, under

semi-aerobic conditions in the dark approximates the differential rate of chlorophyll synthesis characteristic of cells growing photosynthetically under anaerobic conditions at a light intensity of 2,000 ft.-c. For example, in the experiment shown in Figure 2, the value of P is 9 μg per mg dry weight of cell material, as compared to a value of 7.5 μg per mg dry weight of cell material for cells growing photosynthetically at a light intensity of 2,000 ft.-c.

The specific chlorophyll contents of whole extracts and of the chromatophore fractions prepared from cells harvested between one and six hours after the initiation of pigment synthesis under semi-aerobic conditions in the dark are shown in Table 6. The parallelism between the values for extracts and for purified chromatophores is close. The fraction of cell protein represented by chromatophores, calculated from these data, ranged from 36.4 to 47.3%; it was, if anything, slightly higher in the early samples, but the significance of the variation is questionable. The average value for all samples was 41.5%, close to the figure of 45% calculated for cells grown anaerobically at a light intensity of 2,000 ft.-c.

TABLE 6

SPECIFIC BACTERIOCHLOROPHYLL CONTENT OF WHOLE EXTRACTS AND PURIFIED CHROMATOPHORE FRACTIONS FROM *R. rubrum* GROWN SEMI-AEROBICALLY IN THE DARK

Duration (hr) of semi-aerobic growth	Specific chlorophyll: μg per mg protein		Percentage of total cell protein present in chromatophore material
	Whole extract	Purified chromatophores	
1	1.55	3.28	47.3
1.5	2.55	5.51	46.3
2	2.68	7.37	36.4
4	9	22.6	40
6	11.8	31.5	37.5

The specific activities of photophosphorylation and succinic dehydrogenase in purified chromatophores from the same series of samples are shown in Table 7.

TABLE 7

ACTIVITIES OF PURIFIED CHROMATOPHORES PREPARED FROM INITIALLY DEPIGMENTED CELLS OF *R. rubrum* AFTER VARIOUS PERIODS OF GROWTH IN THE DARK UNDER SEMI-AEROBIC CONDITIONS

	Duration (hr) of semi-aerobic growth at time of harvesting and of chromatophore isolation						Average value for purified chromatophores from cells grown at a light intensity of 2,000 ft.-c.*
	1	1.5	2	4	6	6.5	
Specific chlorophyll of chromatophores: μg per mg protein	3.28	5.51	7.37	22.6	31.5	31	22.8
Photophosphorylation:							
$Q_{Pi\ (chlorophyll)}$							
No PMS	1,360	1,175	965	967	847	947	694
PMS	...	...	...	1,450	1,250	...	1,100
$Q_{Pi\ (protein)}$							
No PMS	4.45	6.5	7.1	21.8	26.6	29.3	15.3
PMS	...	...	...	32.8	39.4	...	25
Succinic dehydrogenase:							
$Q_{succ.\ (protein)}$	...	...	11.2	13.6	13.4	13.1	12.9

* From data in Table 3.

Calculated on a chlorophyll basis, the rate of photophosphorylation is highest in the early samples. These early values are far higher than any obtained for chromatophores of photosynthetically grown cells. As the chlorophyll content of the

chromatophores rises, the rate of photophosphorylation falls to a figure only slightly above that found for cells grown photosynthetically at a light intensity of 2,000 ft.-c. Taken as a whole, these figures suggest that even very soon after the initiation of pigment synthesis in the dark, cyclic photophosphorylation is not limited by the amount of chlorophyll in the photosynthetic apparatus. The rate of photophosphorylation calculated on a protein basis shows a quite different trend: there is a linear increase in specific activity as the chlorophyll content of the chromatophores rises (Fig. 3), and the final value obtained after 6 hr of semi-aerobic growth is much higher than any of the values found for photosynthetically grown cells. The specific succinic dehydrogenase activity of the chromatophores, on the other hand, is relatively constant, and of the same magnitude as that found in chromatophores from photosynthetically grown cells (*cf.* Table 5).

Two interpretations of the increase in the specific rate of photophosphorylation as measured on a protein basis are possible. This increase could imply that the chromatophore material formed early in the course of semi-aerobic growth has a low specific activity because it is deficient in one or more of the components required for the dark reactions of cyclic photophosphorylation, and that the specific activity subsequently rises as a result of increased formation of the specific limiting components. Alternatively, one could imagine that the photosynthetic apparatus is formed in association with particulate cell structures devoid themselves of photophosphorylative capacity, and that the purified chromatophores isolated soon after the initiation of pigment synthesis contain a large fraction of such photochemically inactive material. According to this interpretation, the steady rise in the rate of photophosphorylation measured on a protein basis would reflect a relative decrease in the amount of photochemically inactive material in the chromatophore fraction as chlorophyll synthesis proceeds. The constancy of specific succinic dehydrogenase activity in the successive chromatophore fractions would accordingly imply that this enzyme is present in both photosynthetically active and photosynthetically inactive components.

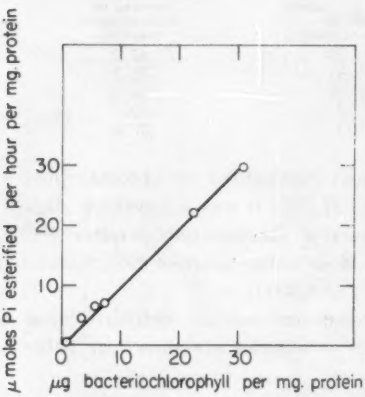


FIG. 3.

It is not possible to decide on the basis of the available information which of these two interpretations is correct. However, the relatively constant fraction of cell protein present in chromatophores throughout the course of semiaerobic pigment synthesis is most easily reconciled with the second interpretation: if the entire photosynthetic apparatus, as well as chlorophyll, were synthesized *de novo* from the start of semi-aerobic growth, the fraction of cell protein localized in the chromatophores should rise continuously.

Summary.—1. The purified chromatophores isolated from cells of *R. rubrum* grown anaerobically at high and low light intensities have markedly different chlorophyll contents, the differences being closely correlated with the differences in

chlorophyll content of the cells from which they were prepared. It is therefore evident that light-induced changes of cellular chlorophyll content reflect mainly changes in the amount of chlorophyll that is incorporated in the photosynthetic apparatus, rather than changes in the amount of chromatophore material in the cell.

2. In chromatophores prepared from cells growing photosynthetically under steady state conditions, the rate of photophosphorylation measured on the basis of chlorophyll content is inversely related to the amount of chlorophyll present in the chromatophores. Measured on the basis of protein content, however, the rate of phosphorylation is practically constant in chromatophores with very different chlorophyll contents. These facts indicate that the capacity for photophosphorylation is not limited by the amount of chlorophyll in the photosynthetic apparatus, but rather by some enzymatic component of the system.

3. When initially depigmented cells of *R. rubrum*, produced under strictly aerobic conditions of growth, are subjected to oxygen limitation in the dark, a rapid synthesis of chlorophyll ensues: the differential rate of chlorophyll synthesis is of the same order as that which occurs during anaerobic growth in the light.

4. The synthesis of chlorophyll under semi-aerobic conditions in the dark is accompanied by the formation of photochemically active chromatophores. The rate of photophosphorylation by such chromatophores as measured on the basis of protein content increases with the chlorophyll content of the cells, eventually attaining a value considerably higher than that characteristic of chromatophores prepared from cells grown under photosynthetic conditions.

* This work was supported by a grant from the National Science Foundation to Professor M. Doudoroff.

[1] Cohen-Bazire, G., W. R. Sistrom, and R. Y. Stanier, *J. Cell and Comp. Physiol.*, **49**, 25 (1957).

[2] Schachman, H. K., A. B. Pardee, and R. Y. Stanier, *Arch. Biochem. and Biophys.*, **38**, 245 (1952).

[3] Newton, J. W., and G. A. Newton, *Arch. Biochem. and Biophys.*, **71**, 250 (1957).

[4] Bergeron, J. A., *Brookhaven Symposium* No. **11**, 118 (1958).

[5] Frenkel, A. W., *J. Am. Chem. Soc.*, **76**, 5568 (1954).

[6] Frenkel, A. W., *Annual Rev. Plant Phys.*, **10**, 53 (1959).

[7] Arnon, D. I., *Nature*, **184**, 10 (1959).

[8] Woody, B. R., and E. S. Lindstrom, *J. Bact.*, **69**, 353 (1955).

[9] Jensen, S. L., G. Cohen-Bazire, T. O. M. Nakayama, and R. Y. Stanier, *Biochem. et Biophys. Acta*, **29**, 477 (1958).

[10] *Methods in Enzymology*, ed. S. P. Colowick and N. O. Kaplan, (New York: Academic Press Inc., 1958), Vol. III, p. 448.

[11] Vernon, L. P., and M. D. Kamen, *J. Biol. Chem.*, **211**, 643 (1954).

[12] Bock, R. M., and N. Ling, *Anal. Chem.*, **26**, 1543 (1954).

[13] *Carnegie Institution of Washington Year Book 1958*, p. 262 (Washington, D.C., 1959), Report of: Bolton, E. T., R. J. Britten, D. B. Cowie, B. J. McCarthy, K. McQuillen, and R. B. Roberts.

[14] Frenkel, A. W., *J. Biol. Chem.*, **222**, 823 (1956).

[15] Geller, D. M., Thesis, Harvard University, 1957.

[16] Fiske, C. H., and Y. Subbarow, *J. Biol. Chem.*, **66**, 375 (1925).

[17] Kearny, E. G., and T. P. Singer, *J. Biol. Chem.*, **219**, 963 (1956).

[18] Glick, D., and C. G. King, *J. Am. Chem. Soc.*, **55**, 2445 (1933).

[19] Lascelles, J., *Biochem. J.*, **72**, 508 (1959).

*INTERACTION OF SOLUBLE RIBONUCLEIC ACID AND MICROSOMES**

By Mahlon B. Hoagland and Lucy T. Comly

THE JOHN COLLINS WARREN LABORATORIES OF THE HUNTINGTON MEMORIAL HOSPITAL OF HARVARD UNIVERSITY, AT THE MASSACHUSETTS GENERAL HOSPITAL, BOSTON, MASSACHUSETTS

Communicated by Joseph C. Aub, October 13, 1960

Transfer (or soluble) RNA (S-RNA) has come to occupy a central position in current views on the mechanism of protein synthesis. Amino acids are activated enzymatically and then attached to specific S-RNA molecules. The resulting compounds—amino acid esters of the 2′ or 3′ ribosyl hydroxyl groups of the terminal adenosyl moieties of RNA chains consisting of some 100 bases—appear to serve as carriers of activated amino acids to the site of peptide bond formation, the ribosomes. It has been suggested that these molecules may act as "adaptors," conferring identity upon the amino acids in the form of specific sequences of nucleotides which may be "recognized" by the ribosomal RNA template through the principle of hydrogen bonding between complementary base pairs.[1, 2] Such a hypothesis envisions S-RNA molecules orienting themselves on the ribosomal RNA template in such a way as to permit contiguous amino acids to condense and then returning to the soluble phase to repeat the cycle.

A number of preliminary observations, using S-RNA labeled in its purine, pyrimidine, or phosphate moieties[3–6] have supported the contention that at least some of the S-RNA accompanies the amino acids into microsomes during the course of amino acid transfer from S-RNA to protein. Bosch *et al.*, furthermore, have more recently fractionated S-RNA on ECTEOLA columns and have found a major component which both binds amino acids and is at least in part incorporated into microsomes.[7]

Observations from our laboratory on the incorporation into microsomes of S-RNA labeled in its terminal adenosyl residue indicated that the rate of its entry was considerably more rapid than the rate of incorporation of a single amino acid into protein; and that the S-RNA incorporation rapidly reached a plateau value while amino acid continued to enter protein.[8] The results were consistent with the conclusion that an amino acid on S-RNA accompanied the terminal nucleotide to which it was attached into the ribosomes. Moldave[9] reached a similar conclusion using a somewhat different system.

The experiments to be presented here attempt to obtain information on the following points. First, if the rapidly reached plateau of S-RNA incorporation into microsomes is due to an equilibrium between entry and exit, it should be possible to displace labeled S-RNA from microsomes (previously labeled with S-RNA) by incubating with unlabeled S-RNA. Such a displacement should be specific for S-RNA, and should have requirements similar to those for amino acid incorporation into protein. Second, if the first point can be established, the *initial rate* of entry of uniformly-labeled S-RNA into ribosomes should give information as to the total quantity of RNA which passes through the ribosomes during the course of incorporation of a given amount of amino acid into protein. Third, if there is a breakdown of S-RNA-amino acid compounds to metabolically active acid-soluble nucleotide-amino acid compounds, the latter might be detected by appropriately sensitive techniques.

Using these three approaches we conclude that a large fraction of the S-RNA pool cycles through the microsomes during the course of amino acid transfer to protein and that fragmentation of the molecules is not detectable.

Materials and Methods.—Preparation of RNA and enzyme fractions: The pH 5 fraction from mouse Ehrlich ascites tumor cells, in which the S-RNA component was labeled *in vivo* with P^{32} and *in vitro* with C^{14} amino acids, was prepared in the following manner. On the 5th day after transplantation of the tumor, a total of 400 μcuries of P^{32} were injected intraperitoneally into 20 tumor-bearing mice in three doses over a period of 2 days. On the 7th day (post-transplantation), the mice were killed by cervical fracture, and the tumor cells were harvested, washed, and lysed according to the methods described by Hecht *et al.*[10] The 105,000 $\times$ *g* supernatant (S_3) fraction and the pH 5 precipitable fraction (pH 5 fraction) derived therefrom were prepared as previously described,[10] except that the final dissolving medium was 0.2 *M* Tris buffer (pH 7.6), 0.005 *M* $MgCl_2$, 0.025 *M* KCl (pH 5 medium). The pH 5 fraction, in about 25 ml of pH 5 medium, was frozen, and precipitated material was centrifuged off and discarded before the next step. The whole pH 5 fraction was then incubated at 37° for 10 min in a total volume of 40 ml with 10 m*M* ATP and 0.1 m*M* valine-C^{14} (containing approximately 14 $\times$ 10^6 cpm). The reaction mixture was poured into 2 volumes of ice-cold distilled water, the pH brought to 5.1 with *M* acetic acid, and the resulting precipitate collected by centrifugation. The precipitate was dissolved in 40 ml of pH 5 medium, diluted as before with water, and reprecipitated at pH 5.1. The final pH 5 fraction was dissolved in 5 to 10 ml of pH 5 medium and frozen.

In all experiments in which this pH 5 fraction was incubated with microsomes, the fraction was centrifuged first at 105,000 *g* for 50 min to insure removal of material which might otherwise accompany microsomes upon their subsequent resedimentation. The amounts and radioactivity of protein and RNA were determined on an aliquot of the pH 5 fraction by methods previously described.[10, 11] Plated protein and RNA samples in all experiments were counted directly and also after shielding with two layers of aluminum foil. The aluminum foil eliminated all of the counts due to C^{14} but permitted the detection of 95 per cent of the P^{32} counts. This served as a method both for quantitating the amount of C^{14} and P^{32} radioactivity on RNA and for correcting the protein radioactivity for small amounts of contamination with P^{32}.

S-RNA labeled with P^{32} and/or C^{14} amino acids was prepared from this or other pH 5 fractions by the usual phenol and alcohol precipitation procedures.[10] S-RNA stripped of its amino acids was prepared by treatment at pH 10 in 0.1 *M* Na_2CO_3 at 37° for one hour.[12]

RNA was prepared from baker's yeast by the method of Monier *et al.*[13] This RNA is similar in physical and chemical properties to S-RNA prepared by centrifugal means from other tissues.

The S_4 fraction (the supernatant fraction from the pH 5 precipitation step) was prepared from Ehrlich ascites tumor cells.[10] It was concentrated to one third of its original volume by lyophilization and dialyzed against medium A before use.

Microsomes were prepared freshly for all experiments from the livers of 80 gm rats fasted 18 hr before use. Adenosine triphosphate (ATP), guanosine triphos-

phate (GTP), phospho-enol pyruvate (PEP), and PEP kinase were the same preparations as those described in earlier publications from this laboratory.

An extract of microsomes was prepared by incubating previously frozen microsomes with 5 volumes of water at 37° for 1 hr, followed by centrifugation at 100,000 *g* for 1 hr.

A nuclear gas flow counter with a Micromil end window was used throughout for counting.

Incubations to determine simultaneous rates of labeling of microsomal RNA and protein by P^{32}-, C^{14}-labeled pH 5 fraction: Microsomes, suspended in 4 ml of medium A,[14] derived from approximately 30 gm. of rat livers, were incubated at 28° in a total volume of 10 to 11 ml with 4 to 4.5 ml of P^{32}-, C^{14}-amino acid-labeled pH 5 fraction (previously centrifuged at 105,000 × *g* as described above), 0.5 m*M* GTP, 0.5 m*M* ATP, 10 m*M* PEP, and 0.5 to 1 mg. PEP kinase. All ingredients except microsomes were mixed and temperature-equilibrated at 28° for 3 min. Microsomes were equilibrated separately and were then mixed with the other ingredients. 1.0 ml aliquots were taken at fixed time points and pipetted into 10 ml of ice-cold medium A in No. 30 Spinco centrifuge tubes. The tubes were immediately filled with medium A, and at the end of the experiment, all tubes were centrifuged at 4° for 90–120 min at 78,000 × *g* in the Spinco Model L preparative ultracentrifuge. The supernatant solutions were discarded and the microsomal pellets analyzed for protein and RNA content and radioactivity. To minimize P^{32} contamination, all protein samples were washed 4 times with hot trichloroacetic acid before plating and were recounted shielded (with aluminum foil) to correct for such possible contamination. Corrections never amounted to more than 10 per cent of the C^{14} radioactivity of the protein samples.

Incubations to determine displacement of P^{32}-S-RNA from previously labeled microsomes: Freshly prepared microsomes were incubated with P^{32}-, C^{14}-amino acid abeled pH 5 fraction for 4–5 minutes at 28° as described above. The whole reaction mixture was then poured into approximately 140 ml of medium A, mixed, divided among 12 No. 40 Spinco tubes, and centrifuged at 105,000 × *g* for 60 min. The S-RNA-labeled microsomal pellets were pooled and resuspended by homogenization in about 5 ml of medium A, and then 0.5 ml of the microsomal suspension was further incubated with 0.15 ml of S_4 fraction, 0.5 m*M* ATP, 0.5 m*M* GTP, 10 m*M* PEP, and 0.5–1.0 mg PEP kinase in a final volume of 1.0 ml. S-RNA or microsomal RNA was added as indicated. At the end of the incubation the tubes were filled with cold medium A and centrifuged at 105,000 × *g* for 90 min. The supernatant solutions were decanted into fresh tubes, 0.5 mg. of yeast S-RNA was added to each tube (to insure the quantitative precipitation of the labeled S-RNA present), and the RNA isolated and prepared for counting. The protein and RNA of the microsomal pellets were quantitated and counted as described above.

Incubations to detect metabolically active acid-soluble fragments of S-RNA: 0.5 to 1.0 mg (protein by dry weight) of the tissue fraction (pH 5 fraction, S_4 fraction frozen microsomes or microsomal extract) was preincubated in a 1.0 ml volume at 37° with S-RNA labeled with leucine-C^{14} or valine C^{14} with the following additions: 0.5 m*M* ATP, 5 m*M* PEP, 0.1 mg PEP kinase, plus or minus 0.5 m*M* GTP. At the designated time points, the reaction mixture was chilled, and to one half

of it (0.5 ml) was added 500 μg of yeast S-RNA (as carrier), and perchloric acid to a final concentration of 0.4 *N*. The RNA of these aliquots was isolated and counted. The remaining half of the reaction mixture was then further incubated for 15 minutes at 37° with 0.5 ml of a mixture containing 0.3 ml of freshly prepared S_2 fraction (the 15,000 *g* supernatant fraction of a rat liver homogenate), 0.5 m*M* ATP, 0.5 m*M* GTP, 5 m*M* PEP, 0.1 mg PEP kinase, and 2.5 m*M* C^{12}-leucine or C^{12}-valine. The reaction was stopped by adding TCA to a final concentration of 4 per cent, and the protein of the samples was washed, weighed, and counted in the usual manner.

This type of incubation procedure thus afforded a simultaneous determination of the rate of disappearance of S-RNA-amino acid, and the capacity of the system to incorporate amino acid into microsomal protein.

Results and Discussion.—The tumor cells were exposed to P^{32} from the 5th to the 7th day after transplantation. The cells approximately double in number during this period.[15] It is known that both the pyrimidine[15] and purine bases[16] of RNA in these cells retain their label for prolonged periods after exposure to labeled precursors; i.e., newly synthesized RNA, like DNA, is conserved. The terminal adenosine moiety of S-RNA, although readily exchangeable with ATP in partially purified ruptured cell preparations[10, 17] apparently does not undergo extensive renewal in whole ascites tumor cells incubated *in vitro*.[16] It would appear safe to conclude that the P^{32} in the S-RNA used in these experiments is distributed generally throughout the newly synthesized molecules.

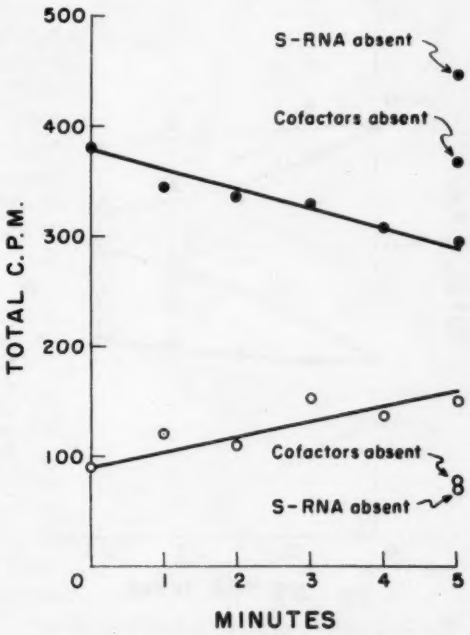


FIG. 1.—Displacement of P^{32}-S-RNA from microsomes in the presence of unlabeled S-RNA and cofactors. Microsomes previously incubated with pH 5 fraction (1.0 ml of which contained 3.1 mg protein, 0.42 mg S-RNA, and 4,700 cpm as P^{32}) and cofactors (ATP, GTP, PEP, and PEP kinase) were reisolated and reincubated with yeast S-RNA (0.2 mg per tube) and cofactors as described in the text. There were 1.8 mg microsomal RNA per tube. Closed circles are radioactivity of microsomal RNA; open circles are radioactivity of soluble RNA.

Displacement of labeled S-RNA from microsomes: Microsomes that have been incubated with P^{32}-S-RNA for brief periods and then reisolated, are found to release soluble P^{32}-RNA upon subsequent incubation with unlabeled S-RNA (Fig. 1). There is a release of counts from microsomal RNA and an accumulation of counts in the supernatant fraction. The release of P^{32}-S-RNA from the microsomes depends upon the unlabeled S-RNA, GTP, S_4 fraction and generating

system. Microsomal RNA cannot replace S-RNA in this displacement reaction (Fig. 2).

It was found that in such displacement reactions, only about 20 to 30 per cent of the P^{32}-S-RNA originally incorporated into the microsomes was recovered in the soluble phase during the second incubation. It was also noted that the reaction proceeded considerably more slowly than the initial S-RNA incorporation reaction (compare the rate in Fig. 1 with the rate of incorporation of P^{32}-S-RNA in Fig. 4).

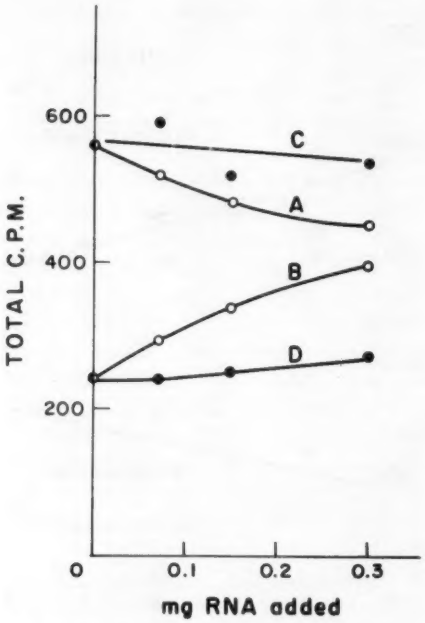


FIG. 2.—Displacement of P^{32}-S-RNA from microsomes as a function of the kind and concentration of unlabeled RNA added. Conditions were the same as those in Figure 1. (The original pH 5 fraction used for labeling the microsomes contained 11.0 mg protein, 1.1 mg S-RNA, and 8,900 cpm as P^{32}.) *Curves A and B:* radioactivity of microsomal RNA and soluble RNA, respectively, following incubation of microsomes (2.9 mg microsomal protein per final incubation tube) with yeast S-RNA. *Curves C and D:* radioactivity of microsomal RNA and soluble RNA, respectively, following incubation of microsomes with ascites tumor microsomal RNA.

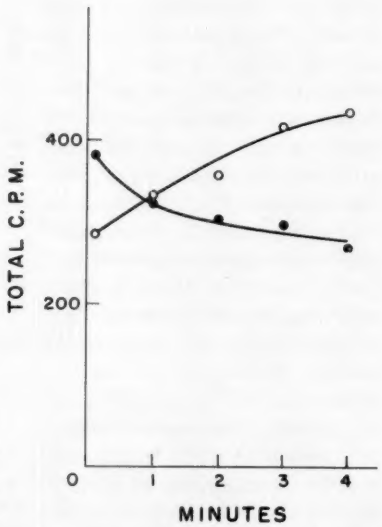


FIG. 3.—Comparison of ability of reisolated unlabeled microsomes to incorporate labeled S-RNA (open circles) and of reisolated labeled microsomes to incorporate unlabeled S-RNA (closed circles). Conditions were as described in the text and in the previous figures.

It was suspected that the difference in rate might be due to the fact that the reisolation of the microsomes had damaged them, thus rendering them less capable of incorporating S-RNA, or, alternatively, that the reisolated microsomes were saturated with S-RNA, while the original ones contained unoccupied sites. Either situation might be expected to lead to a slower rate of equilibration of S-RNA with reisolated microsomes which had previously been incubated with S-RNA. These possibilities were tested as follows: half of a suspension of microsomes was incu-

bated with pH 5 fraction (P^{32}-S-RNA), the other half with unlabeled pH 5 fraction. Both fractions were prepared in an identical manner from ascites tumor cells, half of which had been exposed *in vivo* to P^{32} as described. The microsomes were reisolated by centrifugation at 100,000 × *g* for 1 hr. The labeled half was then further incubated with unlabeled pH 5 fraction, while the unlabeled microsomes were incubated with labeled pH 5 fraction, the conditions otherwise being identical. Thus, the capacity of reisolated microsomes for acquiring P^{32}-S-RNA could be compared to their capacity to replace labeled S-RNA with unlabeled S-RNA. The results (Fig. 3) indicate a rough equivalence between the P^{32}-S-RNA gained by unlabeled reisolated microsomes and the P^{32}-S-RNA lost by labeled reisolated microsomes. Longer periods of incubation did not enhance the yield of S-RNA from reisolated microsomes. We therefore conclude that the reisolation of previously incubated microsomes has in some way impaired their capacity for carrying out the S-RNA incorporation reaction.

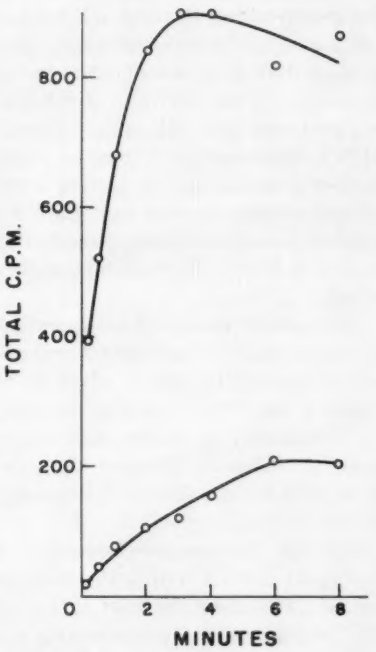


FIG. 4.—Simultaneous incorporation of P^{32}-S-RNA into microsomal RNA and C^{14}-amino acid into microsomal protein in presence of P^{32}-, C^{14}-amino acid-labeled S-RNA. Each time point aliquot contained microsomes (1.9 mg RNA) and 0.24 mg of S-RNA (originally containing 1,400 cpm as P^{32} and 210 cpm as C^{14}-amino acid) and cofactors as described in the text. *Upper curve:* P^{32} incorporation into microsomal RNA; *lower curve:* C^{14} incorporation into microsomal protein.

The dependence of RNA release from microsomes upon ATP and GTP must be interpreted with some caution however. Siekevitz and Palade[18] have shown that microsomes in the presence of concentrations of ATP and GTP similar to those used in our experiments readily lose RNA to the soluble phase. This is thought to be due to the Mg^{++}-complexing properties of these polyphosphates, Mg^{++} being essential to the integrity of ribosomal RNA. That our results represent a different phenomenon is suggested by the facts that in our experiments (1) ATP and GTP are added with one mole of Mg^{++} per 2 moles of PO_4; (2) there is no spectrophotometrically detectable loss of RNA from the microsomal pellets during our incubations (which are at lower temperatures and of shorter duration than those of Siekevitz and Palade); (3) the release of RNA depends upon the S_4 fraction and thus appears to be an enzymatic process; (4) the release of RNA depends upon the addition of S-RNA, microsomal RNA being ineffective.

The displacement of S-RNA from microsomes, taken in conjunction with the S-RNA incorporation studies to be described in the next section would permit a tentative conclusion that the S-RNA-dependent release of labeled soluble RNA

from microsomes is a manifestation of the same process as the initial incorporation of labeled S-RNA into the microsomes.

Simultaneous incorporation of P^{32}-S-RNA into microsomal RNA and C^{14}-amino acid into protein: Incubation of microsomes with pH 5 fraction whose S-RNA component is labeled with P^{32}- and C^{14}-amino acids results in rapid incorporation of the P^{32} into microsomal RNA and of C^{14}-amino acid into microsomal protein (Fig. 4). The transfer of amino acid to protein is usually complete in 6 to 8 min. The incorporation of the S-RNA into microsomal RNA reaches its maximum in 2 to 3 min. In most experiments, the maximal level of P^{32} incorporation remains constant during the remainder of the incubation period as amino acid accumulates in protein. Thus, the ratio of S-RNA incorporated into amino acid rises rapidly to a peak and then falls off as amino acid continues to accumulate in protein and S-RNA (presumably) begins to return to the soluble phase. When complete transfer of amino acid to protein is reached at 6 min (Fig. 4), the ratio ceases to fall and remains constant from the 6th to the 8th min. In four experiments of this type, the microsomes were saturated when 1 to 5 per cent of their RNA was occupied by S-RNA. This amount of S-RNA was 29 to 35 per cent of the amount added.

The ratio of the rate of incorporation of P^{32} into microsomal RNA to the rate of incorporation of C^{14} into microsomal protein is not grossly different from the P^{32}/C^{14} ratio in the S-RNA used to effect the transfer. For example, in the experiment of Figure 4, the P^{32}/C^{14} ratio in the starting S-RNA was 6.7 and the rate ratio was 7.2. Similarly, in another experiment, the starting ratio was 5.7 and the rate ratio 7.6. This we interpret to mean that within the limits of error, essentially all of the S-RNA molecules accompany the attached amino acids into the particles during the transfer reaction.

The high background (zero time) level of radioactivity appears to be due to binding of S-RNA during the prolonged centrifugations at 4° and is difficult to reduce. It is about the same as the radioactivity of incubated controls from which GTP or GTP, ATP, and generating system have been omitted.

The nature of the linkage of S-RNA to microsomal RNA is a subject for further study. The adaptor hypothesis would require the linkage so be chiefly hydrogen bonding between complementary sequences of bases on each of the two types of RNA, ribosomal and soluble. We can thus far say only that the S-RNA attached to microsomes is indistinguishable from microsomes in sedimentation properties and that its binding to microsomes requires GTP. Bloemendal *et al.*[19] report in preliminary studies that the binding of S-RNA to microsomal RNA appears to be covalent in nature, being resistant to EDTA and 8 *M* urea.

One may ask whether the incorporation of S-RNA into ribosomes depends on the presence of amino acids on the former. Can a molecule as small as that of an amino acid influence the binding of a molecule whose molecular weight is 200 to 300 times greater? Figure 5 is an experiment which attempts to answer this question. Here S-RNA labeled with P^{32} and C^{14}-amino acid was incubated with microsomes and the rate of incorporation was compared to that of a similar S-RNA preparation from which the amino acids had been stripped. Note that in spite of the fact that there is good incorporation of amino acid into protein with natural RNA and no incorporation with "stripped" RNA, the rate of incorporation of P^{32} is

similar with both preparations. This result suggests that the bound amino acids are not necessary for the S-RNA incorporation reaction. The experiment is, however, not entirely conclusive since it is possible that small amounts of free amino acids present in the microsomes could be activated and transferred to the "stripped" S-RNA during the course of incubation.

Possible formation of acid-soluble utilizable fragments of S-RNA: Although the above results suggest that most of the S-RNA molecule accompanies the amino acid into the ribosomes, we had done a series of experiments earlier to detect acid-soluble nucleotide-amino acid fragments of S-RNA which could be used by ribosomes for protein synthesis. The results are briefly presented here because they support, as negative data, our general conclusion.

The principle of the experiments was as follows: S-RNA, labeled with C^{14}-amino acid, was incubated with various cellular fractions which were known to be involved in the incorporation reaction but which in themselves were incapable of accomplishing the final step of incorporating the C^{14}-amino acid into protein. All such fractions (pH 5 fraction, S_4 fraction, frozen microsomes, microsomal extract) were found to degrade S-RNA-amino acid. At intervals during this reaction, freshly prepared microsomes (and the other components of the amino acid incorporating system) were added, and the incubation continued for a fixed period. The reaction was carried out in the presence of C^{12}-amino acid to prevent the reincorporation of *free* C^{14}-amino acid released from S-RNA. If utilizable acid-soluble nucleotide-amino acid compounds had been formed during the initial incubation, one would expect them to be used by the competent microsomes during the second incubation. This would be detected as a nonparallelism between the rate at which S-RNA was degraded to acid-soluble fragments and the incorporating ability of the subsequently added microsomes.

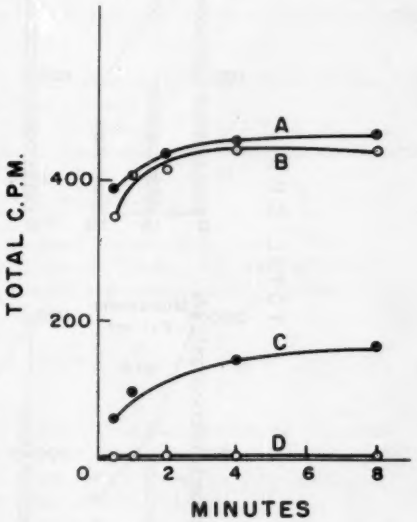


FIG. 5.—Simultaneous incorporation of P^{32}-S-RNA into microsomal RNA (curves A and B) and C^{14}-amino acid into microsomal protein (curves C and D). Curves A and C were obtained by using P^{32}-, C^{14}-amino acid-labeled S-RNA, curves B and D by using the same S-RNA preparation after removal of amino acids by treatment at pH 10.

The results of a series of such experiments were all similar: regardless of the fraction used to attack S-RNA-amino acid, whether in the presence or absence of GTP, ATP and generating system, the amount of amino acid subsequently incorporated into protein was always proportional to the amount of amino acid still remaining on acid-insoluble S-RNA. A group of such experiments are shown in Figure 6. We therefore conclude that the amino acid must remain bound either to intact S-RNA molecules or to fragments of such molecules of sufficiently high

molecular weight as to precipitate under acid conditions in order to be incorporated into ribosomal protein. Bosch *et al.*[7] have also looked for labeled polynucleotides resulting from incubation of their active component of S-RNA with various cellular fractions and have found no evidence that such are formed.

The observations of Scott cited earlier[16] on intact ascites tumor cells incubated

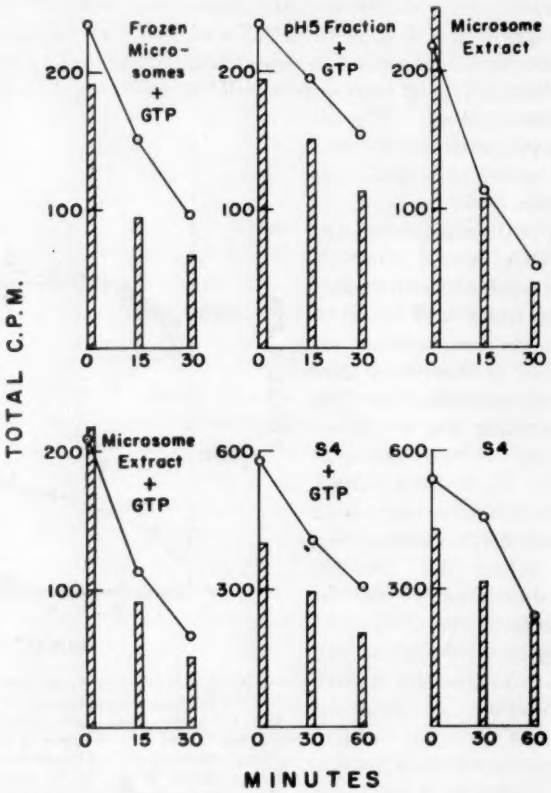


FIG. 6.—The rate of disappearance of C^{14}-leucine labeled S-RNA, in the presence of various cell fractions, and the resulting capacity of the system to incorporate the amino acid into microsomal protein. Conditions as described in the text. The curve represents the quantity of C^{14}-leucine-S-RNA; the bars indicate the extent of incorporation obtained in the microsomes subsequently added. In the experiments where S_4 fraction was assayed, C^{14}-valine-S-RNA was used.

in vitro support the conclusion that neither the whole S-RNA molecule nor the terminal adenosyl residue is renewed at a significant rate compared to the rate at which the S-RNA molecule must turn over the attached activated amino acids to account for the net synthesis of protein. The stability of S-RNA in the face of rapid protein synthesis would appear to mean that S-RNA is used repeatedly for protein synthesis without intermediate breakdown.

Summary.—By using P^{32}-, C^{14}-amino acid-labeled S-RNA it has been possible to study the simultaneous incorporation of S-RNA into microsomal RNA and of C^{14}-amino acid into microsomal protein. The kinetics are consistent with the concept that the admission of an amino acid into peptide linkage requires a large fraction of the associated S-RNA molecule to become firmly and transitorily bound to microsomal RNA. This is further substantiated by experiments in which microsomes previously equilibrated with P^{32}-S-RNA are shown to release the latter on further incubation with unlabeled S-RNA and cofactors necessary for protein synthesis. Evidence that there is no detectable fragmentation of S-RNA to smaller utilizable pieces is also presented.

We wish to thank Dr. Jesse Scott for his interest and valuable criticism during the course of this study.

* This is publication No. 1025 of the Cancer Commission of Harvard University. The work was supported by a grant from the U.S. Public Health Service.

[1] Crick, F. H. C., *Symposia Soc. Exptl. Biol.*, **12**, 138 (1958).

[2] Hoagland, M. B., P. C. Zamecnik, and M. L. Stephenson, in *A Symposium on Molecular Biology*, ed. R. E. Zirkle (Chicago: Univ. of Chicago Press, 1959), p. 105.

[3] Von der Decken, A., and T. Hultin, *Exptl. Cell. Res.*, **15**, 254 (1958).

[4] Hoagland, M. B., *Rec. trav. chim.*, **77**, 623 (1958).

[5] Hoagland, M. B., *Proc. 4th Intern. Congr. Biochem.*, Vienna (1958).

[6] Bosch, L., H. Bloemendal, and M. Sluyser, *Biochim. et Biophys. Acta*, **34**, 272 (1959).

[7] Bosch, L., H. Bloemendal, and M. Sluyser, *Biochim. et Biophys. Acta*, **41**, 444 (1960).

[8] Zamecnik, P. C., *Harvey Lectures*, **54**, 256 (1960).

[9] Moldave, K., *J. Biol. Chem.*, **235**, 2365 (1960).

[10] Hecht, L. I., P. C. Zamecnik, M. L. Stephenson, and J. F. Scott, *J. Biol. Chem.*, **233**, 954 (1958).

[11] Siekevitz, P., *J. Biol. Chem.*, **195**, 549 (1952).

[12] Zamecnik, P. C., M. L. Stephenson, and J. F. Scott, these PROCEEDINGS, **46**, 811 (1960).

[13] Monier, R., M. L. Stephenson, and P. C. Zamecnik, *Biochim. et Biophys. Acta*, **43**, 1 (1960).

[14] Keller, E. B., and P. C Zamecnik, *J. Biol. Chem.*, **221** 45 (1956).

[15] Scott, J. F., and E. B. Taft, *Biochim. et Biophys. Acta*, **28**, 45 (1958).

[16] Scott, J. F., unpublished data.

[17] Herbert, E., *J. Biol. Chem.*, **231**, 975 (1958).

[18] Siekevitz, P., and G. E. Palade, *J. Biophys. and Biochem. Cytol.*, **7**, 619 (1960).

[19] Bloemendal, H., L. Bosch, and M. Sluyser, *Biochim. et Biophys. Acta*, **41**, 454 (1960).

ANALYSIS OF SEQUENCE PATTERNS IN RIBONUCLEASE, I. SEQUENCE VECTORS AND VECTOR MAPS

BY FRANK LANNI*

DEPARTMENT OF MICROBIOLOGY, EMORY UNIVERSITY, ATLANTA

Communicated by E. L. Tatum, October 27, 1960

Patterns of amino acid sequence in polypeptides and proteins have been recognized previously.[1–7] Reported patterns include repeats of identical or similar sequences, interpolations into otherwise familiar sequences, inversions of sequence, and periodic or interdependent spacing of certain residues. Recently discovered regularities in the composition of nucleic acids,[8] whose structure is currently

imagined to relate intimately to that of proteins, encourage an intensified search for additional evidence of patternization in proteins.

In this paper, we consider the chain of beef pancreatic ribonuclease, whose proposed sequence formula[9] is reproduced in Table 1, and examine the arrangement of the amino acid residues by a new method, *vectorial analysis.* The basic analytical tool is the *sequence vector,* which is primarily a device for representing the comparative order of the residues in two or more subsequences, regardless of the orientation of the peptide bonds connecting the residues. We introduce two kinds of vectors, beginning with the cruder, but currently indispensable, *analogue sequence vectors.* We show that (1) structural analogies can be discerned among moderately extensive subsequences that together comprise more than half the molecular chain; (2) if a reference vector is assigned to a certain one of these subsequences, and if vectors for other, structurally similar, sequences are then deduced, the resulting incomplete *vector map* shows several inversions of vector, or *vector breaks;* (3) these vector breaks generally appear to occur near half-cystine (CyS) residues; (4) with slight modification and extension, the vector map obtained in this fashion predicts with considerable precision the mutual orientation of certain repeating dipeptides. Hence, the introductory analogue mapping procedure provides a basis for assigning the biochemically more precise, but analytically more elusive, *dipeptide sequence vectors.* Earlier, but less comprehensive, analyses of sequence patterns in ribonuclease have been published.[3, 6]

Analogue Sequence Vectors.—Analysis of sequence patterns in the ribonuclease chain would preferably begin with repeating subsequences if there were enough of these and if they were sufficiently long. The use of repeating dipeptides for this purpose presents unsolved problems, which are discussed in a later section. The chain contains only three repeating tripeptides, each represented twice, and not a single repeat of a longer sequence. We have therefore investigated an alternative method of analysis, based on postulated analogies between residues rather than on identities.

Classification of amino acid residues: The method to be described rests on the following tentative classification of amino acid residues into three groups according to structural, functional, and other similarities:

1. *Dominant group:* CyS and the cyclic residues (histidine, phenylalanine, proline, and tyrosine; tryptophane is absent from the molecule). The unusual significance of these residues for chain structure and for analysis of sequence patterns is suggested by (*a*) the structural resemblance of several of the cyclics to the purine and pyrimidine bases of nucleic acids,[2] (*b*) the interdependent spacing of several of the cyclics in the chains of polypeptides and proteins,[2, 3] (*c*) the familiar chain-bending property of paired CyS residues and proline, (*d*) the apparently high immunity of each of these residues to substitution by others in the evolution of polypeptides and proteins,[10] and (*e*) the results of the present analysis.

2. *Subdominant group:* arginine and lysine, aspartic and glutamic acids, and the amides of the latter two. The basic and acidic residues are grouped together because of their ionizability. The amides are included because of their structural resemblance to the acids.

3. *Minor group:* the remaining residues.

It is important to stress that the foregoing classification is strictly an analytical

device, to be judged by the results, and may be only one of many equally useful ways of initiating a study of sequence patterns. The classification is not suggested at the moment to have biochemical significance outside the context of the present analysis.

In comparing sequences, we shall place greatest emphasis on the dominant residues, next on the subdominant residues, and least on the minor residues. For the most part, the minor residues will be regarded as equivalent wherever they occur. We do not intend, however, to equate freely the residues in either the dominant or subdominant group. Instead, we shall attempt to "feel out" the permitted interchanges within these groups. We exclude, however, interchanges of residues classified in different groups, since the freedom thereby gained would weaken the force of the analysis. The proposed restrictions on interchange accord with known differences of composition between beef and sheep pancreatic ribonucleases,[11] as discussed below. In the initial comparisons, we shall deal exclusively with sequences of not less than four residues in order to minimize distraction by fortuitous resemblances.

Some preliminary comparisons: According to the foregoing orientation, the following octapeptide sequences of ribonuclease show impressive similarities:[12]

	1	8
	*lys.glu.*thr.ala.ala.ala.*lys.*PHE	
8	9	16
(PHE)	*glu.arg.*ser.thr.ser.ser.*aspN.*HIS	
84	85	92
(CyS)	*arg.glu.*ser.thr.gly.ser.*lys.*TYR	

Each sequence begins with a couplet of one basic and one acidic residue, followed by four minor residues; a basic residue or an amide; and a cyclic. Each is bounded at the left by the *N* terminus or a dominant residue. The three octapeptide sequences thus appear to represent a well-defined natural unit, which occurs with the same overall orientation at three locations in the molecule.

The structural relatedness of these octapeptides and the obvious possibility, still necessarily speculative, that they arose from a common ancestral sequence are supported by known interchanges among the minor residues and also among the subdominant residues when *beef* proteins and polypeptides are compared with corresponding proteins and polypeptides of other species. Thus, the threonine residue at position 3 in beef pancreatic ribonuclease is replaced by a serine residue in the corresponding sheep enzyme.[11] The alanine and serine residues at the *adjacent* positions 8 and 9, respectively, of the A chain of beef insulin are replaced by threonine and glycine residues, respectively, in the insulins of other species.[13] Interchanges involving alanine, serine, threonine, and glycine residues at two adjacent positions occur also in the tobacco mosaic viruses.[14] The minor residues of the octapeptides hence appear to constitute a compact evolutionary group. As for the subdominant residues, the lysine residue at position 37 in beef ribonuclease is replaced by a glutamic acid residue in the sheep enzyme; and an asparagine residue, perhaps at position 101, is also replaced by a glutamic acid residue.[11] Interchanges of arginine and lysine residues occur repeatedly in comparisons of beef polypeptides

and proteins with those of other species;[15] none has yet been reported for ribonucleases. To make free use of these and other known interchanges would render our analysis arbitrary. We intend, therefore, to be conservative in the process of aligning sequences for the deduction of vectors.

The ribonuclease chain contains six tetrapeptide sequences, each with a subdominant couplet, which may be compared with the *N*-terminal sections of the octapeptides:

27 30	61 64
aspN.gluN.met.met	*lys.aspN*.val.ala
33 36	66 69
arg.aspN.leu.thr.*lys.asp*	*lys.aspN*.gly.thr.*aspN.gluN*
35 38	68 71

The sequence 35–38 alone possesses a couplet of acidic and basic residues, similar to that of the octapeptides. In each of two sequences, 33–38 and 66–71, a tetrapeptide of interest might be read from either end. Rather than conclude that each of these sequences contains a point of inversion within its span, we prefer the more conservative conclusion that only the sequence 35–38 matches (inverted) the *N*-terminal sections of the octapeptides. In the absence of a dominant residue, we place intermediate confidence in this alignment.

There are also four sequences, larger than tripeptides, that may be compared with the *C*-terminal sections of the octapeptides:

20 25	100 97
ala.ser.ser.ser.*aspN*.TYR	thr.thr.*lys*.TYR
52 48	124 120
ala.leu.ser.*glu*.HIS	val.ser.ala.*asp*.PHE

Of these, only the sequences 20–25 and 97–100 (inverted) contain a basic residue or an amide next to the cyclic, and only these may be aligned confidently with the *C*-terminal sections of the octapeptides.

Sequence vectors: In any two aligned subsequences, the constituent residues may be ordered in the same direction in the molecular chain, or the respective orders may be mutually inverted. Either of these order relations is conveniently described by arrows, called *sequence vectors*, which are written along the subsequences and directed to conform to the order of the residues regardless of the peptide-bond orientation. These vectors serve primarily descriptive and analytical functions and do not require any enabling postulate about the biochemical significance of order relations. As illustrated below, the analytical utility of sequence vectors stems from the ease with which they can be recorded, compared, combined, subdivided, extended, viewed as a collection, evaluated quantitatively, and revised.

An analogue sequence-vector map: We proceed to develop stepwise a vector map based on analogies. As will be seen, the analysis makes negligible use of repeating dipeptides, which are deliberately kept in reserve for a later check. For ease of reference, the steps below are lettered to correspond with the lettered vectors in Table 1, where the suggested degree of confidence in a vector increases with the length and density of its shaft.

(a) As a primary standard of reference, a sequence vector directed away from the *N* terminus is assigned to sequence 1–8. This vector orientation will be called *positive*, its reverse *negative*. Two similarly directed vectors will be called *parallel*, two oppositely directed ones *antiparallel*.

(b) In accordance with the previous discussion, vectors are deduced for the sequences 9–16, 20–25, 35–38 (inverted), 85–92, and 97–100 (inverted). Assignment of a negative vector at 97–100 is strengthened by the vicinal CyS in the comparison:

$$\underset{22}{\overset{\longrightarrow}{\text{ser.ser.}\textit{aspN}\text{.TYR.CyS}}}\underset{26}{}$$

$$\underset{96}{\text{CyS.TYR.}\textit{lys}\text{.thr.thr}}\underset{100}{}$$

Confidence in this alignment is further increased by the chemical similarity of the minor couplets. The vector pattern, showing three breaks, now reads:

$$\underset{1\text{–}16}{\longrightarrow}\ \underset{20\text{–}25}{\longrightarrow}\ \underset{35\text{–}38}{\longleftarrow}\ \underset{85\text{–}92}{\longrightarrow}\ \underset{97\text{–}100}{\longleftarrow}$$

(c) This pattern is reinforced considerably and extended by the comparison:

$$\underset{28}{}\underset{34}{\overset{\longleftarrow}{\textit{gluN}\text{.met.met.}\textit{lys}\text{.ser.}\textit{arg.aspN}\text{.(35–38)}}}$$

$$\underset{98}{\overset{\longleftarrow}{\textit{lys}\text{.thr.thr.}\textit{aspN}\text{.ala.}\textit{gluN.lys}}}\underset{104}{}$$

Confidence in the alignment is increased by the agreement with prior vectors, by the complete reciprocity of basic residues and amides, and by the minor couplets. Proceeding conservatively on the principle of "inertia of polarity," *i.e.*, assuming a continuous vector unless a break is clearly indicated, we extrapolate local vectors over the aligned sequences and write the extended vector pattern:

$$\underset{1\text{–}16}{\longrightarrow}\ \underset{20\text{–}25}{\longrightarrow}\ \underset{28\text{–}38}{\longleftarrow}\ \underset{85\text{–}92}{\longrightarrow}\ \underset{97\text{–}104}{\longleftarrow}$$

(d) A striking example of mutually inverted sequences is noticed in:

$$\underset{23}{\overset{\longrightarrow}{\text{ser.}\textit{aspN}\text{.TYR.CyS.}\textit{aspN.gluN}\text{.met.met.}\textit{lys}}}\underset{31}{\overset{\longleftarrow}{}}$$

$$\underset{67}{\overset{\longrightarrow}{\textit{aspN}\text{.gly.thr.}\textit{aspN.gluN}\text{.CyS.TYR.}\textit{gluN}\text{.ser}}}\underset{75}{\overset{\longleftarrow}{}}$$

The central portions of these sequences show complete reciprocity between the two amides. With a minor consolidation at 17–19, we write the expanded vector pattern:

$$\underset{1\text{–}25}{\longrightarrow}\ \underset{28\text{–}38}{\longleftarrow}\ \underset{67\text{–}70}{\longrightarrow}\ \underset{73\text{–}75}{\longleftarrow}\ \underset{85\text{–}92}{\longrightarrow}\ \underset{97\text{–}104}{\longleftarrow}$$

Role of CyS residues: The pattern just written indicates at least five vector breaks in the map for ribonuclease. Inspection of the still unmapped sequences

shows that (1) three of the breaks occur very near a CyS residue, (2) at least one CyS is present in each gap where a break is suspected, (3) CyS is the only type of residue consistently present in these gaps, and (4) the longest unmapped sequence, 39–66, must undergo an *odd* number of breaks and indeed contains *three* CyS residues. Further, there appears to be a systematic alternation of vector configurations, the available vectors being *afferent* at odd-numbered CyS residues and *efferent* at even-numbered ones.

The notion of a fundamental distinction between odd and even CyS residues is supported by inspection of the vicinal residues at each of the eight CyS residues:

I	V	II	VI
TYR.CyS.*aspN*	*gluN*.CyS.TYR	*arg*.CyS.*lys*	*asp*.CyS.*arg*
III	VII	IV	VIII
val.CyS.ser	ala.CyS.TYR	ala.CyS.*lys*	ala.CyS.*glu*

It can be seen that (1) the CyS-odd neighbors include all of the three tyrosines and the two amides, (2) the CyS-even neighbors include all of the four basic and the two acidic residues, (3) no odd CyS has a strongly ionized neighbor, whereas each even CyS has one or two. Within each of the two groups of CyS residues there is a tendency for alternate members to show the greatest similarity.

In further support of the distinction between odd and even CyS residues, inspection of the four *cystine* bridges in the molecule[16] shows that each consists of an odd and even CyS, as follows:

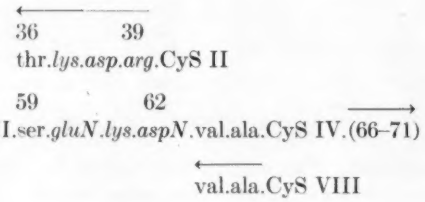
I:VI; II:VII; III:VIII; IV:V

It appears, therefore, that a few apparently reasonable initial assumptions about the structural significance of individual residues have revealed an impressive patternization of the ribonuclease chain, fully consistent with the independent chemical considerations just mentioned.

Additional vectors: With this encouragement, we venture beyond the earlier bounds of confidence to deduce vectors in the remaining gaps. As will be seen, many subsequences in these spans can be fitted with vectors that conform to a simple rule of inversion at each CyS residue.

(e) At sites 27, 39, 66, 71, 76–83, and 105–109, existing vectors are extrapolated to the nearest CyS without serious conflict anywhere, provided that a distinction is maintained between odd and even CyS residues. Extrapolation at 93–95 is avoided for a reason given below.

(f) Evidence of a negative vector in the span 59–64, hence, of a break at CyS IV, is given by the comparison:



The tetrapeptide sequences 36–39 and 59–62 contain the only two occurrences in ribonuclease of a subdominant triplet, which is flanked in each sequence by a chemically similar minor residue. Assignment of a negative vector to 59–64 is consistent with the alignment of the tetrapeptides and also with that of the CyS-vicinal val.ala dipeptides.

(g) There remain two large unmapped spans, 41–57 and 111–124, which resemble each other in overall composition and differ notably from other sections of the chain. The unusually high density of proline, histidine, phenylalanine, and valine residues in these spans gives them a somewhat "amorphous" character and makes them difficult to map by analogy with sequences already mapped. The following, admittedly insecure, alignments suggest that some portions, at least, of the two spans are mutually parallel and have a positive vector:

48 55
HIS.*glu*.ser.leu.ala.*asp*.val.*gluN*

120 124
PHE.*asp*.ala.ser.val

111 113
(CyS).*glu*.gly.*aspN*

$\longrightarrow$ $\longrightarrow$
8 9 83 82
PHE.*glu* (CyS).*asp*.thr

The listed sequences contain the only three occurrences in ribonuclease of a cyclic-vicinal acidic residue, the only two occurrences of a CyS-vicinal acidic residue, and the only two occurrences of a tripeptide of an acidic residue and an amide separated by a minor residue (note reciprocity between acids and amides). The rarity of these configurations justifies the cautious assignment of a positive vector to the aligned subsequences.

Extrapolation of local vectors over the remaining gaps would involve assignment of a positive vector to each of the mutually inverted tripeptides:

92 94 113 115
TYR.PRO.*aspN* *aspN*.PRO.TYR

It seems preferable to face this difficulty directly by turning to an alternative method for analyzing sequence patterns.

Dipeptide Sequence Vectors.—Repeating dipeptides: The ribonuclease chain contains 30 distinct *kinds* of dipeptides of the form X.Y which repeat either with the same chemical polarity or as the sequence isomer Y.X or both (Table 2). Twenty-four of these repeating dipeptides are 2-fold repeaters (occur twice in the chain), five are 3-fold repeaters, and one is a 4-fold repeater. Thus, there are 67 total occurrences of repeating dipeptides. This count excludes vectorially neutral dipeptides such as ala.ala and ser.ser. The chain contains three different symmetrical tripeptides of the form X.Y.X. Here, the overlapping isomeric dipeptides X.Y and Y.X are each counted once and included in the total.

Among the 30 repeaters, there are 10 (*parallel repeaters*) for each of which all the

occurrences have the same chemical polarity in the chain. The remaining 20 (*inverted repeaters*) are each represented by the mutually inverted forms X.Y and Y.X. The occurrences of the various repeaters are intermingled in the chain without superficially obvious order.

Principles of mapping: In the vectorial analysis of sequences by means of

TABLE 1

SEQUENCE-VECTOR MAPS FOR BEEF PANCREATIC RIBONUCLEASE*

| | | |

as many basically distinct vector maps as there are ways of making 29 independent assignments. The number of such maps is 2^{29}, or about 5×10^8. The number would, of course, be larger if there were reasons for taking into account any residues not included in the repeaters.

The problem of vectorial analysis via dipeptides is to discover in this vast atlas one or more maps that seem to make "sense," i.e., present a discernible pattern of vector breaks and/or conform to a set of *a priori* rules or restrictions. Proceeding with the repeating dipeptides as the primary data, one might, for example, look for maps that minimize the vector breaks or maximize them or place them systemati-

TABLE 2

QUANTITATIVE EVALUATION OF VECTOR-MAP HYPOTHESES AGAINST REPEATING DIPEPTIDES

		Credit Units for Hypothesis		
		1	2	3
Repeater	Occurrences	Single vector	Vector break at each CyS	Modified analogue map
ala, asp	2			
ala, aspN	2	2		2
ala, cyS	3	3		3
ala, gluN	2		2	2
ala, ser	2	2	2	2
ala, val	3		3	3
arg, cyS	2		2	2
arg, glu	2			
arg, ser	2		2	2
aspN, gluN	2	2		2
aspN, gly	2			2
aspN, lys	2	2		2
aspN, pro	2			2
aspN, ser	2	2	2	2
aspN, thr	3			3
aspN, val	2		2	2
cyS, lys	2	2	2	2
cyS, tyr	3		3	3
glu, ser	2	2	2	2
gluN, lys	2	2	2	2
gluN, ser	2			
gly, thr	2			2
his, val	2	2	2	2
lys, ser	2		2	2
lys, thr	2			
lys, tyr	2		2	2
pro, tyr	2			2
pro, val*	3			3
ser, thr*	4			
ser, tyr*	2			
Totals	67	21	30	53
Dipeptide score (%)	(100)	31	45	79

* Occurrences include symmetrical tripeptide of form X.Y.X.

cally at certain residues. We plan to deal more extensively with this general problem elsewhere. In the following sections, we shall mainly (a) examine the relation of dipeptide sequence vectors to the analogue map previously derived and (b) use the analogue map as a restrictive guide to an interesting dipeptide vector map.

Quantitative evaluation of hypotheses: Any *a priori* hypothesis regarding the complete sequence-vector map for ribonuclease may be tested separately against each of the 30 distinct sets of repeating dipeptides. The hypothesis will be said to

fit a set (repeater) if and only if the sequence vector demanded by the hypothesis for one member of the set gives a consistent result for all members. In general, an hypothesis will fit some sets and not others. For any hypothesis, the *dipeptide score* is computed by crediting two, three, or four units, respectively, for each fully consistent 2-fold, 3-fold, or 4-fold repeater, and expressing the sum as a percentage of the possible maximum (67 units for a perfect hypothesis). This simple device gives some recognition to differences in the random likelihood of fit, depending on the size of a set, yet avoids needless sophistication.

We proceed to check a few map hypotheses against the repeating dipeptides.

Hypothesis 1: Single vector: By way of introduction, it is instructive to imagine that a single, unbroken vector spans the entire chain. This is equivalent to inquiring about the mutual orientation of the several occurrences of each repeater as they stand in the chain. As shown in Table 2, the dipeptide score for the hypothesis is 31 per cent (21 credit units out of 67). A value closer to 50 per cent might be expected for a system of randomly oriented dipeptides. The low score arises mainly from the fact that, for the majority (15/24) of 2-fold repeaters, the two occurrences are mutually inverted. Improvements on the hypothesis should, therefore, be measured from 31 per cent rather than from a higher value.

Hypothesis 2: Vector break at each CyS residue: In effect, this hypothesis extends the previously derived analogue map by extrapolating all vectors to the nearest CyS. The dipeptide score of 45 per cent (Table 2) is discouraging at first sight. Analysis shows, however, that many of the misfits are concentrated in interesting regions of two kinds: (1) the short span between CyS IV and V, and (2) the three proline-containing spans that were left unmarked in the original analogue map. To a large extent, improvements in these spans can be achieved independently of improvements between CyS IV and V. We shall tackle them in one stroke.

Hypothesis 3: Analogue map, modified and extended: Starting with the original, incomplete analogue map, we (1) reverse the short vector between CyS IV and V (this is the only actual change in the original map) and (2) complete the map by extrapolating all vectors in such a way as to locate breaks at each of the four proline residues and at CyS I, III, VI, VIII.

The completed map is shown immediately to the right of the columns of residues in Table 1. Next to it are indicated the fully consistent repeaters (marked by dipeptide sequence vectors) and the inconsistent ones (marked by circles). The dipeptide score of 79 per cent (Table 2) shows that there has been a remarkable improvement. The new hypothesis fits all the repeaters fitted by either of the previous hypotheses, plus six other repeaters. The remaining misfits involve six repeaters, with a total of 14 occurrences. Whichever vectors are assigned arbitrarily to these six repeaters, only seven dipeptides in a total of 67 remain unfitted. Hence, 90 per cent of the 67 dipeptides are actually fitted by the hypothesis.

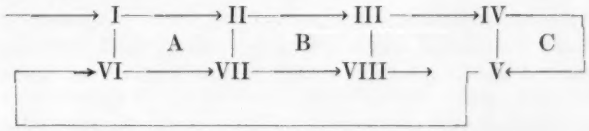
The new map has several attractive systematic features. First, the eight vector breaks are assigned conservatively, and in equal numbers, to only two kinds of residues, CyS and proline. Second, these residues are functionally similar in their helix-distorting property, which gives them unusual importance in defining the three-dimensional configuration of the chain. Third, all of the four proline residues of the chain are made sites of vector inversion. Fourth, although vector breaks are

assigned to only four of the eight CyS residues, these four are not randomly chosen; instead, they are paired as I:VI and III:VIII in two *cystine* residues. Two other CyS residues, II and VII, which also make up one cystine residue, are located very near a proline-sited vector break.

The two remaining CyS residues, IV and V, bound the short span where we found it desirable to reverse the originally assigned analogue sequence vector in order to improve the dipeptide score. There are several ways of rationalizing the need for this reversal. First, chemical analysis of the sequence in this span met with difficulty;[9] perhaps the proposed sequence formula is incorrect. Second, when regarded as a unit, the interval is seen to have considerable sequential symmetry:

IV V
CyS.*lys.aspN*.gly.thr.*aspN.gluN*.CyS

A positive or negative analogue sequence vector might be written with almost equal justification; indeed, one might even be tempted to locate a vector break at the center of the span. Finally, the interval is structurally unique (in ribonuclease) in that it exists as a tight loop closed by a disulfide bridge between the very two CyS residues that bound the interval. The following diagram of the four disulfide bridges[16] illustrates this feature:



Here, the arrows represent the *chemical* order of the residues proceeding from the *N* terminus to the *C* terminus. As can be seen, the molecule contains three economically constructed disulfide rings, labeled *A*, *B*, and *C*. Ring *C*, involving Cys IV and V, is the only one that makes use of a single disulfide bridge. This ring can be traversed without change of chemical polarity; the other two cannot. For future concern, it may be noted that certain sequence-vector maps, including the previously considered map with a vector break at each CyS, make it possible to traverse all three disulfide rings without change of sequence vector. The present map (Hypothesis 3) misses only in the very short regions separating CyS II and VII from the nearby proline residues. These considerations may perhaps furnish a clue to the chemical significance of vectors and vector breaks.

There are many ways of revising the present map in its imperfect regions to produce a perfect fit of the repeating dipeptides. Since the choice among these ways is arbitrary at present, and since Hirs, Moore, and Stein[9] have suggested that their proposed sequence formula for ribonuclease is best regarded as a working hypothesis, we prefer to let the matter rest until additional guides become available. In appropriate cases, chemical error in the ordering of as few as two adjacent residues can affect the dipeptide score by 10 per cent.

The symmetrical tripeptides X.Y.X, two of which are left unfitted, present problems that have not yet been resolved. If, as we have tacitly assumed, the residues themselves are the actual points of vector inversion, and if no special exemption is made, a break would be required at the central residue of each sym-

metrical tripeptide. If, instead, the breaks were to be localized in the bonds between residues, a vector could pass through such a tripeptide effectively unbroken, since the two breaks would cancel. One might even explore the possibility of excluding systematically from consideration one of the two overlapping dipeptides that can be read.

Discussion.—The sequence-vector maps shown in Table 1 imply an unusual degree of order in the mechanisms that integrate the synthesis of the ribonuclease chain. Long-range order is indicated by the span of some of the individual analogue sequence vectors, by the structural analogies between subsequences far removed in the chain, and by the pattern of location and displacement of vector breaks in both kinds of vector map. Short-range order is indicated mainly by the concordance of individual dipeptide sequence vectors to the local vector field. It appears that each residue must (*a*) be consistent with the vicinal residue on either side and (*b*) form with its neighbors a subsequence that is consistent with subsequences elsewhere in the chain. Consistency in the sequence relations is an intimate mixture of identity and analogy.

The nature of the integrative mechanisms remains a matter of conjecture. One possibility, suggested especially by the analogue map, is that the detailed structure of the chain is specified jointly by two functionally distinct mechanisms, one of which defines an amino acid sequence *pattern* and partially restricts thereby the choice of residues, the other of which makes the final selection from the remaining choices. Schwartz[4] has suggested a duality of this kind for adrenocorticotropic hormone.

Going deeper, one might look to the nucleic acids, the presumed ultimate determinants of protein specificity, for compositional regularities that might directly explain the regularities found in ribonuclease. The problem of structural patterns in proteins would then become in part or entirely a problem of patterns in nucleic acids. It seems noteworthy that recent considerations[8] of the composition of nucleic acids have suggested two notions: first, that large portions of a given polynucleotide strand are mutually complementary (cf. ref. 17); second, that the local composition of nucleic acids is specified jointly by two sets of genetic mechanisms, one of which imposes initial restrictions on choice (among nucleotides), the other of which makes the final selection. The striking concordance of these independently derived ideas—intra-chain complementarity and dualistic determination—with the sequence patterns in ribonuclease suggests that some formal aspects of the structural relation of proteins and nucleic acids may be within reach.

It is difficult to resist the obvious (and orthodox) postulate that the order discovered in ribonuclease stems from a matching order in an underlying nucleic-acid determinant. Structural analogies among subsequences of the nucleic acid would suffice to explain both kinds of sequence-vector map. Mutation, with restrictions on the interchangeability of nucleotides at given sequence sites in the nucleic acid,[8, 18] would tend to develop and preserve sequence analogies both in the nucleic acid and in the protein, once the structural basis (sequence repeats, etc.) for these analogies had appeared in the nucleic acid during its evolution. Such mutational restrictions could also explain any observed restrictions on the interchangeability of amino acids.[10, 18]

Some applications and extensions of the technique of vectorial analysis come

quickly to mind. One is to explore the transfer of sequence vectors between ribonuclease and other proteins and polypeptides. Another is to compare functionally homologous proteins or polypeptides from different species with especial attention to the interchanging amino acids. Preliminary studies along both of these lines have given encouraging results. More than anything else, it seems desirable to refine the analytical tools. The excellent check of the analogue mapping procedure against the repeating dipeptides suggests that our initial classification of amino acids is on the right track and encourages a search for more restrictive and precise rules of analogy. Discovery of analogue rules that can be applied at the level of dipeptides would help to interpret the agreement between the two kinds of sequence vector and would also increase the density of useful information in a chain. It may not be too much to look forward to the tabulation of standard sequence vectors.

Several workers, Šorm[7] perhaps more explicitly than others, have recently surmised that the sequences both of proteins and of nucleic acids, far from being chaotic, will prove to be governed by a small set of rules. In our opinion, one of the most valuable products of the present analysis is the support that it gives to this optimism.

We are gratefully indebted to many individuals, and especially to Drs. Stanford Moore, John M. Reiner, and E. L. Tatum, for reading one or more drafts of the manuscript and for valuable suggestions and criticisms.

Several months after the manuscript was completed except for some minor revisions, we made some discoveries that confirm the validity of vectorial analysis and the conclusion that the ribonuclease chain exhibits considerable long- and short-range order. Manuscripts describing the new results are in preparation.

* The author's research is supported in part by grant E-857 from the National Institute of Allergy and Infectious Diseases, U.S. Public Health Service.

[1] Anfinsen, C. B., and R. R. Redfield, *Advances Protein Chem.*, **11**, 1 (1956); *Symposium on Protein Structure*, ed. A. Neuberger (New York: John Wiley & Sons, 1958). Many earlier references are cited.

[2] Schwartz, D., these PROCEEDINGS, **41**, 300 (1955).

[3] Schwartz, D., *Nature*, **181**, 769 (1958).

[4] Schwartz, D., *ibid.*, **183**, 464 (1959).

[5] Šorm, F., B. Keil, V. Holeyšovský, V. Knesslová, V. Kostka, P. Mäsiar, B. Meloun, O. Mikeš, V. Tomášek, and J. Vaněček, *Coll. Czech. Chem. Comm.*, **22**, 1310 (1957). Many earlier references are cited.

[6] Šorm, F., and B. Keil, *Comm. Czech. Chem. Comm.*, **23**, 1575 (1958).

[7] Šorm F., *Coll. Czech. Chem. Comm.*, **24**, 3169 (1959).

[8] Lanni, F., *Perspectives Biol. Med.*, **3**, 418 (1960).

[9] Hirs, C. H. W., S. Moore, and W. H. Stein, *J. Biol. Chem.*, **235**, 633 (1960).

[10] Lanni, F., unpublished survey of the literature.

[11] Anfinsen, C. B., S. E. G. Åqvist, J. P. Cooke, and B. Jönsson, *J. Biol. Chem.*, **234**, 1118 (1959).

[12] The customary abbreviations for the residues are employed except that the amide groups of asparagine and glutamine residues are indicated simply by the suffix *N*. The typography used in the text for the various symbols is chosen to facilitate sequence comparisons.

[13] Harris, J. I., F. Sanger, and M. A. Naughton, *Arch. Biochem. and Biophys.*, **65**, 427 (1956).

[14] Niu, C. I., and H. Fraenkel-Conrat, *Arch. Biochem. and Biophys.*, **59**, 538 (1955).

[15] du Vigneaud, V., H. C. Lawler, and E. A. Popenoe, *J. Am. Chem. Soc.*, **75**, 4880 (1953);

Tuppy, H., in *Symposium on Protein Structure*, ed. A. Neuberger (New York: John Wiley & Sons,, 1958), p. 66; Harris, J. I., *Nature*, **184**, 167 (1959).

[16] Spackman, D. H., W. H. Stein, and S. Moore, *J. Biol. Chem.*, **235**, 648 (1960).

[17] Platt, J. R., these PROCEEDINGS, **41**, 181 (1955); Silk, M. H., and A. O. Hawtrey, *Nature*, **184**, 1484 (1959).

[18] Freese, E., these PROCEEDINGS, **45**, 622 (1959).

---

*RECONSTITUTION OF THE DINITROPHENOL-SENSITIVE ATP-ADP EXCHANGE REACTION OF OXIDATIVE PHOSPHORYLATION**

BY CHARLES L. WADKINS† AND ALBERT L. LEHNINGER

DEPARTMENT OF PHYSIOLOGICAL CHEMISTRY, THE JOHNS HOPKINS SCHOOL OF MEDICINE, BALTIMORE MARYLAND

Communicated October 10, 1960

Intact rat liver mitochondria[1] as well as fragments of mitochondrial membranes capable of oxidative phosphorylation[2] catalyze an exchange reaction between ATP and ADP which is inhibited by 2,4-dinitrophenol (DNP). The exchange enzyme has been postulated to catalyze the terminal reaction of oxidative phosphorylation by which ATP is formed.[1–5] The ATP-ADP exchange reaction is inhibited by dinitrophenol only in freshly prepared particles capable of phosphorylation; aging of the particles causes no loss of activity of the exchange but results in loss of its DNP-sensitivity.[2] Similarly, azide has no inhibitory effect on the ATP-ADP exchange, but abolishes its sensitivity to DNP.[2] The stability of the ATP-ADP exchange enzyme made possible its extraction in soluble form[2] and its purification to a high degree.[6] The soluble form of the enzyme is completely insensitive to DNP.[2, 6]

These findings thus show that DNP-sensitivity is not intrinsic but is conferred on the ATP-ADP exchange in fresh mitochondria or membrane fragments because it is in functional equilibrium with an earlier enzymatic reaction of the respiratory energy coupling mechanism which is sensitive to DNP, from which it can be dissociated by aging, azide, or physical separation.

This paper describes the restoration of DNP-sensitivity to the soluble, DNP-insensitive ATP-ADP exchange enzyme separated from rat liver mitochondria, by recombining it with mitochondrial membrane fragments prepared by the action of digitonin.[7] Such "recombination," which is specific, indicates that the soluble ATP-ADP exchange enzyme we have isolated is a participant in the mechanism of respiratory energy-coupling and differentiates it from other mitochondrial enzymes catalyzing ATP-ADP exchanges[2] which are not relevant to oxidative phosphorylation.

Methods.—The ATP-ADP exchange rate was measured with P^{32}- or C^{14}-labeled ADP using the paper chromatographic separation described before.[2] Initial incorporation rates under conditions of substrate saturation were measured. Phosphorylating digitonin particles from rat liver mitochondria were prepared according to Devlin and Lehninger.[7] Soluble ATP-ADP exchange enzyme was prepared according to Wadkins and Lehninger.[2] The purification of the ATP-ADP ex-

change enzyme[6] will be described in detail elsewhere; it involves ammonium sulfate fractionation and chromatography on a column of DEAE-cellulose.

Restoration of DNP-sensitivity of the soluble ATP-ADP exchange enzyme by "recombination" with normal digitonin fragments: Data from several experiments in Figures 1–4 and in Table 1 show the initial rates of the ATP-ADP exchange, and its inhibition by 5×10^{-4} *M* DNP, in given aliquots of the soluble exchange enzyme and of fresh digitonin fragments, first tested separately and then incubated together in the presence of 0.001 *M* $MgCl_2$. It is seen that the soluble enzyme is completely insensitive to 5×10^{-4} *M* DNP, but the ATP-ADP exchange in the digitonin fragments is inhibited by about 65 per cent. (No further inhibition can be produced by increasing the DNP concentration.) When the two types of enzymes are then incubated together as a mixture, the expected sensitivity to DNP of the combined activity can be calculated to be about 20 per cent. However, the observed inhibition of the combined enzymes was about 80 per cent, or much larger than expected if the two species of exchange enzyme acted completely independently of each other in the reaction medium. It is thus evident that DNP-sensitivity has been conferred on that portion of the total ATP-ADP exchange reaction which is catalyzed by the added soluble exchange enzyme.

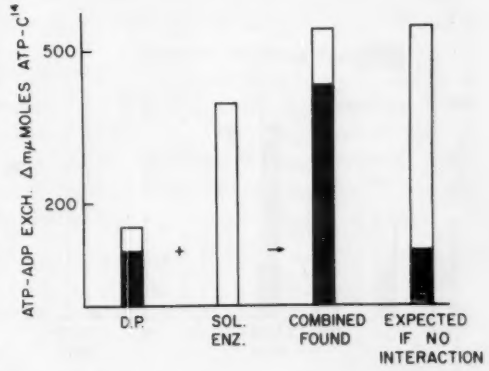


FIG. 1.—"Recombination" of soluble ATP-ADP exchange enzyme with particles. Test details as in Table 1. Shaded area represents that portion of the ATP-ADP exchange activity which is sensitive to dinitrophenol.

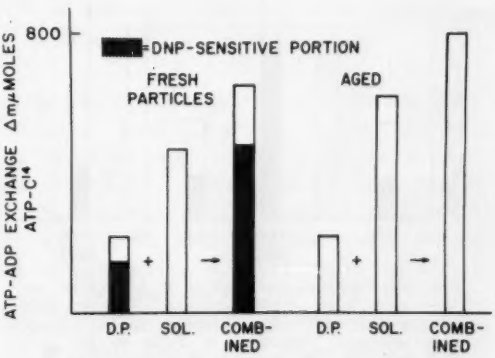


FIG. 2.—Effect of aging of digitonin particles on "recombination" phenomenon. Details as in Table 1. The particles were aged 24 hours at 2°.

The conferral of DNP-sensitivity on the inherently insensitive soluble ATP-ADP exchange enzyme, by adding it to the DNP-sensitive digitonin fragments, is not due simply to an increase in ADP and a decrease in ATP concentration in the medium caused by stimulation of ATP-ase activity of digitonin particles by the DNP, an action which might decrease the ATP-ADP exchange rate through Michaelis-Menten effects. Such an explanation is ruled out since (1) the exchange

reaction rate is measured with short reaction times (3 minutes) over which less than 3 per cent of the ATP in the system is split to ADP + P_i in the absence of DNP and less than 8 per cent in the presence of DNP, and (2) the initial concentrations of ATP and ADP in the test system (0.01 *M* ATP; 0.006 *M* ADP) are so high that even quite large changes in the concentration or ratio of ATP and ADP do not affect the exchange rate. Thus maximum rates of the exchange are given by as low as 0.003 *M* ATP in the presence of ADP varying from 0.002 *M* to 0.010 *M* and by 0.003 *M* ADP in the presence of any concentration of ATP from 0.002 *M* to 0.015 *M*. The limiting capacity of the exchange assay system for maximum rates is thus not remotely approached by the ATP-ase activity of the preparations tested. The conferral of DNP-sensitivity on the soluble exchange enzyme thus is brought about by an interaction of the soluble exchange enzyme and the insoluble digitonin fragments in a specific manner to reconstitute a functional relationship to the DNP-sensitive reaction. The specificity of this effect was demonstrated by the failure of digitonin particles to confer DNP-sensitivity on other soluble enzymes capable of catalyzing ATP-ADP exchange reactions. Soluble adenylate kinase catalyzes an ATP-ADP exchange,[2] but this was found not to become DNP-sensitive on addition to fresh digitonin particles.

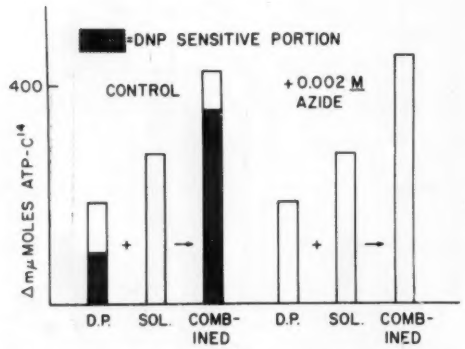


FIG. 3.—Effect of azide on "recombination." Details as in Table 1. Azide was added at 0.002 *M*.

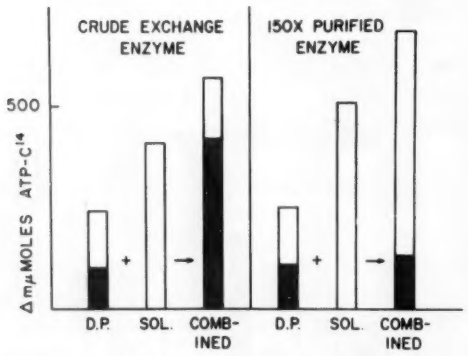


FIG. 4.—Effect of purification of ATP-ADP exchange enzyme on "recombination." Shaded area represents the dinitrophenol-sensitive ATP-ADP exchange activity. Details as in Table 1. 90 μgm. protein 150-fold purified exchange enzyme was added where shown.

Effect of aging digitonin particles on reconstitution: Data in Figure 2 show that the incubation of digitonin particles previously aged 24 hr or longer at 2° (to inactivate the ability for oxidative phosphorylation) with the soluble ATP-ADP exchange enzyme results in a system in which the ATP-ADP exchange is essentially insensitive to DNP. It is therefore clear that conferral of DNP-sensitivity on the soluble ATP-ADP exchange enzyme is specific and depends on the competence of the digitonin particles in catalyzing oxidative phosphorylation.

Effect of azide on recombination phenomenon: Earlier experiments showed that azide does not inhibit the ATP-ADP exchange reaction in either fresh or aged digitonin particles or in the soluble enzyme; however, the presence of 0.002 *M* azide abolishes the DNP-sensitivity of the exchange in fresh digitonin particles. Data in Figure 3 show that 0.002 *M* azide also abolishes the DNP-sensitivity of the combination of soluble exchange enzyme with fresh digitonin particles in agreement with expectation if the recombination is specifically associated with the coupling mechanism.

Effect of purification of the soluble ATP-ADP exchange enzyme on its ability to recombine: The preceding experiments were carried out with preparations of the soluble ATP-ADP exchange enzyme which are relatively crude and purified to only a limited extent (10–20 fold above its activity in intact mitochondria). More recently, the enzyme has been purified approximately 150 fold by ammonium sulfate fractionation, followed by chromatography on DEAE-cellulose columns. It

TABLE 1

"RECOMBINATION" OF SOLUBLE ATP-ADP EXCHANGE ENZYME AND FRESH DIGITONIN PARTICLES TO RESTORE DNP-SENSITIVITY

System*	Rate of ATP-ADP Exchange ATP (C^{14}) (mμmoles)
Zero time	0
Digitonin particles	300
Digitonin particles + DNP	100
Soluble exchange enzyme	500
Soluble exchange enzyme + DNP	490
Particles + soluble exchange enzyme	750
Particles + soluble exchange enzyme + DNP	250
Particles + soluble exchange enzyme + DNP + azide	750

* Each reaction system contained 0.012 *M* ATP, 0.006 *M* ADP (C^{14}) (48,000 CPM), and 0.001 *M* Mg^{++} (pH 6.8) in 0.5 ml volume. Fresh digitonin particles (450 μgm protein) and soluble exchange enzyme (1500 μgm protein) were added where indicated. The incubation period was 3 min at 30°C. DNP was added at 5×10^{-4} *M*; azide at 0.002 *M*.

was found that such fractionation of the soluble exchange enzyme causes loss of its ability to recombine with digitonin particles. Data in Figure 4 compare the DNP-sensitivity of the crude and purified soluble exchange enzyme preparations when they are "recombined" with digitonin fragments. It is seen that a crude sample of the soluble enzyme "recombines" with restoration of DNP-sensitivity, whereas a highly purified fraction obtained from the crude sample by ammonium sulfate fractionation shows no evidence of recombination, i.e., DNP-sensitivity is not conferred on it. Purification of the exchange enzyme by relatively mild procedures thus causes loss of its ability to recombine with the digitonin particles; this suggests that a cofactor for recombination is required. Other explanations are discussed below.

Discussion.—The "recombination" of the DNP-insensitive soluble ATP-ADP exchange enzyme with phosphorylating digitonin particles to reconfer DNP-sensitivity is fully consistent with and provides further support for a general mechanism of respiratory energy coupling postulated earlier:[2–5]

$$(1)\ \text{Carrier} \sim \text{X} + \text{P}_\text{i} \rightleftarrows \text{P} \sim \text{X} + \text{Carrier}$$
$$(2)\ \text{P} \sim \text{X} + \text{E} \rightleftarrows \text{P} \sim \text{E} + \text{X}$$
$$(3)\ \text{P} \sim \text{E} + \text{ADP} \rightleftarrows \text{ATP} + \text{E}$$

where Carrier $\sim$ X is the chemical species of the electron carrier in which is conserved oxido-reduction energy, and X and E are group-transferring enzymes. E is the enzyme catalyzing the ATP-ADP exchange reaction, which has another active site reactive with P $\sim$ X. Many considerations suggest that DNP interferes with reaction (1) to cause decomposition of Carrier $\sim$ X. Such an action accounts for uncoupling of oxidative phosphorylation, stimulation of ATP-ase, and inhibition of the ATP-P_i^{32} exchange and could also account for inhibition of the ATP-ADP exchange by lowering the steady-state concentration of intermediates P $\sim$ X or P $\sim$ E or both, since these are postulated to be in equilibrium with the DNP-sensitive Carrier $\sim$ X.

The fact that the DNP-insensitive soluble ATP-ADP exchange enzyme (reaction (3)) regains DNP-sensitivity on addition to phosphorylating digitonin particles normally capable of P:O ratios of 1.5–2.0 indicates that the digitonin particles contain a considerable number of incomplete "assemblies" of coupling enzymes, from which the terminal ATP-ADP exchange enzyme has become detached during isolation. The sites normally binding this enzyme are evidently still intact and capable of "rebinding" the soluble form of the enzyme in a sufficiently specific manner as to bring the exchange enzyme into the proper situation to become reactive with the preceding enzyme X in the lipoprotein membrane which contains the coupling enzymes. Other studies to be reported indicate that this enzyme is indeed rather easily detached, not only by acetone treatment, but also by manipulation of ionic strength and specific anion effects.

The failure of 150-fold purified soluble ATP-ADP exchange enzyme to recombine with the digitonin particles in such a way as to confer DNP-sensitivity requires special comment. It is possible that the purification procedure selected the "wrong" ATP-ADP exchange enzyme from the crude preparations, which contain minor amounts of adenylate kinase, protein phosphokinase, and possibly other phosphotransferases also capable of catalyzing ATP-ADP exchanges, but which are insensitive to DNP. However, this seems unlikely because the purification procedure yielded relatively little loss of total ATP-ADP exchange activity but complete loss of activity for "recombination." A second explanation is that on purification, the second active site of enzyme E, necessary for its binding to X, may have become damaged without damage to the nucleotide-binding sites catalyzing the ATP-ADP exchange reaction. The third explanation, which is the simplest, is that the purification procedures removed a second non-dialyzable protein component necessary for rebinding the ATP-ADP exchange enzyme to the "empty" sites in the digitonin particle. This could be either a specific "cementing" protein not having enzymatic activity or it could be a preceding enzyme in the coupling sequence, such as the enzyme X which participates in reactions (1) and (2). It is possible that certain respiratory assemblies in the digitonin particles are incomplete with respect to *both* X and E, which must be "rebound" sequentially to confer DNP sensitivity on the isolated exchange enzyme. The enzyme X could have become separated from E during the 150-fold purification of E. In the following paper[8] is described a protein factor (called M-factor) which has been found to increase the fraction of the ATP-ADP exchange enzyme present in untreated digitonin particles which is sensitive to DNP. It has characteristics therefore which suggest it to be equivalent to enzyme X and thus to be necessary for rebinding of

highly purified exchange enzyme E. Preliminary experiments to test this hypothesis suggest that if it is correct, the sequence and molar ratio of these fractions are quite critical for successful "recombination" of highly purified ATP-ADP exchange enzyme.

These experiments also show the general feasibility of reconstructing some features of this "solid-state" enzyme system starting from protein components of known enzymatic capability to restore at least a portion of the kinetic and mechanistic complexity of the energy-coupling process.

Summary.—Addition of the 20-fold purified dinitrophenol-insensitive, soluble, mitochondrial ATP-ADP exchange enzyme to phosphorylating digitonin fragments of the mitochondrial membranes causes "recombination" of the soluble enzyme to those sites in the energy-coupling mechanism, so that dinitrophenol-sensitivity of the ATP-ADP exchange is largely restored. The "recombination" occurs only if the particles are capable of oxidative phosphorylation; no conferral of dinitrophenol sensitivity occurs with aged particles or in the presence of azide. Other enzymes showing ATP-ADP exchange do not show dinitrophenol sensitivity. Purification of the ATP-ADP exchange enzyme to about 150-fold results in loss of the ability to "recombine"; it is suggested a second protein necessary for "rebinding" is lost during fractionation.

The findings also indicate the feasibility of reconstituting the complex reactions of oxidative phosphorylation by combining soluble and insoluble elements in a specific manner.

* Supported by grants from the National Institute of Health, the National Science Foundation, the Nutrition Foundation, Inc., and the Whitehall Foundation.

† Senior Research Fellow, U.S. Public Health Service.

[1] Wadkins, C. L., *J. Biol. Chem.*, in press.

[2] Wadkins, C. L., and A. L. Lehninger, *J. Biol. Chem.*, **233**, 1589 (1958).

[3] Lehninger, A. L., C. L. Wadkins, C. Cooper, T. M. Devlin, and J. L. Gamble, Jr., *Science*, **128**, 450 (1958).

[4] Lehninger, A. L., C. L. Wadkins, and L. F. Remmert, in *Ciba Symposium on Cell Metabolism*, (London: J. and A. Churchill, Ltd., 1959), p. 130.

[5] Lehninger, A. L., in Symposium on "Current Topics of Interest in Biochemistry," *Fed. Proc.*, in press.

[6] Wadkins, C. L., *Fed. Proc.*, **18**, 346 (1959). Full data to be published.

[7] Devlin, T. M., and A. L. Lehninger, *J. Biol. Chem.*, **233**, 1589 (1958).

[8] Wadkins, C. L., and A. L. Lehninger, these PROCEEDINGS, **46**, 1582 (1960).

*A FACTOR WHICH INCREASES THE DINITROPHENOL-SENSITIVITY OF THE ATP-ADP EXCHANGE REACTION OF OXIDATIVE PHOSPHORYLATION**

By Charles L. Wadkins† and Albert L. Lehninger

DEPARTMENT OF PHYSIOLOGICAL CHEMISTRY, THE JOHNS HOPKINS SCHOOL OF MEDICINE

Communicated October 10, 1960

The preceding report showed that the mitochondrial enzyme catalyzing the ATP-ADP exchange reaction,[1–6] which in its soluble purified state is insensitive to dinitrophenol,[2] can be recombined again with mitochondrial membrane fragments to restore the dinitrophenol sensitivity[7] which this reaction possesses in intact mitochondria.

This communication describes a soluble protein factor (called M-factor), obtained from extracts of mitochondria, which is a component in conferring DNP-sensitivity on the ATP-ADP exchange reaction in digitonin particles. Although the ATP-ADP exchange activity of fresh digitonin particles of rat liver mitochondria is inhibited by 5×10^{-4} *M* dinitrophenol, this inhibition is usually not complete, is rather variable in extent,[2] and cannot be increased by increasing the DNP concentration. However, addition of M-factor to such particles greatly increases the fraction of the total ATP-ADP exchange activity of the particles which is sensitive to DNP.

Data from two typical experiments in Table I and a third presented graphically in Figure 1 show that the soluble dialyzed M-factor protein contains no significant ATP-ADP exchange activity itself and has no effect on the rate of the ATP-ADP exchange reaction of rat liver digitonin particles *in the absence of DNP*. However this fraction greatly increases the sensitivity of the ATP-ADP exchange of the particles to 5×10^{-4} DNP, an effect which is most prominent in those preparations of digitonin particles in which the initial sensitivity of the exchange to DNP is relatively low. Increasing concentrations of M-factor produce increasing sensitivity to DNP; a half-maximum effect is given by approximately 5–10 micrograms protein nitrogen of the preparation. The M-factor effect is abolished by heating the soluble fraction 10 min at 80°. The DNP-sensitivity induced by M-factor is completely abolished by 0.002 *M* azide, as is the unstimulated DNP-sensitivity of the exchange.

The M-factor effect is specific; it is not given by proteins such as bovine serum albumin, yeast hexokinase, or other mitochondrial proteins. The M-factor activity is retained for some days in frozen solutions; further storage causes inactivation.

Using the increase in DNP-sensitivity of the ATP-ADP exchange as a semi-quantitative assay method, M-factor has been purified over 40 fold by isoelectric precipitation and ammonium sulfate fractionation, starting either from phosphate extracts of acetone-dried rat liver mitochondria or from ammonium sulfate extracts of fresh, undried mitochondria. High ionic strength evidently detaches M-factor readily from mitochondria. A typical preparation is made as follows: Freshly prepared and washed rat liver mitochondria (~2500 mg mitochondrial protein) were extracted at 0° with 50 ml of 0.30 *M* ammonium sulfate adjusted to pH 7.5. After centrifuging at 9000 × *g* for 15 min, the clear yellow supernatant was dialyzed for

16 hr against 0.1 *M* Tris-HCl buffer pH 7.4. The pH was then lowered to 5.5 and the precipitated material removed. Solid ammonium sulfate was added to the supernatant solution and the fraction precipitating between 50 and 70 per cent saturation was collected, dissolved in 0.05 *M* Tris buffer pH 7.4 and dialyzed. Such preparations contain little or no ATP-ADP exchange activity, no ATP-P_i^{32} exchange, adenylate kinase or ATP-ase nor do they stimulate ATP-ase activity of digitonin particles in the presence or absence of DNP or Mg^{++}. However, preparations of M-factor at this stage do contain significant C-factor activity,[8] i.e., they promote the ATP-induced contraction of rat-liver mitochondria swollen in the presence of reduced glutathione.

At least two modes of action of M-factor appear possible in increasing the DNP-sensitivity of the ATP-ADP exchange reaction. M-factor may serve as a specific "cement" protein enabling the ATP-ADP exchange enzyme to be bound to specific sites on the digitonin particles in such a way that the exchange enzyme is reactive with presumably adjacent enzyme molecule(s) which catalyze an earlier DNP-sensitive stage of energy-coupling and with which the exchange enzyme is normally reactive in a sequential fashion. If this "cementing protein" is missing, the exchange enzyme is still capable of catalyzing the exchange but in a "disconnected" manner, so that intermediates in the exchange reaction are no longer capable of interacting with the preceding steps in energy coupling. A similar explanation has been offered by Pinchot[9] for the role of a heat-stable polynucleotide which causes increased binding of a soluble heat-labile component of the coupling mechanism to the respiratory particles obtained from *Alcaligenes faecalis*.

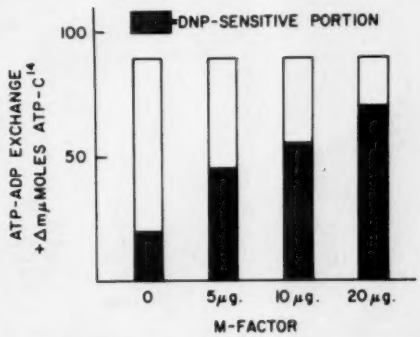


FIG. 1.—Increase of DNP sensitivity of the ATP-ADP exchange reaction of digitonin particles. Fresh digitonin particles (45μg N) and the indicated amounts (protein N) of M factor were used. Other reaction conditions were those described in Table 1.

Alternatively, M-factor may itself be an intermediate enzyme in the sequence of reactions which comprise the energy-coupling cycle of oxidative phosphorylation, acting between the terminal DNP-insensitive ATP-ADP exchange enzyme and the preceding DNP-sensitive intermediate. In this event, M-factor would presumably be a group-transferring enzyme through which decomposition of an early intermediate by DNP could cause substantial lowering of the steady-state concentration of a high-energy intermediate in the ATP-ADP exchange reaction. The following reaction sequence, in which E is the ATP-ADP exchange enzyme and M is M-factor, illustrates such a role:

$$\text{Carrier} + \text{M} \xrightarrow[\text{transfer}]{\text{electron}} \text{Carrier} \sim \text{M}$$

$$\text{Carrier} \sim \text{M} + \text{P}_i \rightleftarrows \text{Carrier} + \text{P} \sim \text{M}$$

$$\text{P} \sim \text{M} + \text{E} \rightleftarrows \text{E} \sim \text{P} + \text{M}$$

$$\text{E} \sim \text{P} + \text{ADP} \rightleftarrows \text{ATP} + \text{E}$$

In this formulation, M-factor is suggested to be the component reacting with the carrier to form the primary, DNP-sensitive, high-energy complex Carrier $\sim$ M. Further work is of course necessary to determine whether or not M-factor is in fact an intermediate enzyme.

It appears possible that M-factor may be involved in the conferral of DNP-sensitivity on the soluble, DNP-insensitive ATP-ADP exchange enzyme when it is "recombined" with digitonin fragments, as described in the preceding paper.[7] *Crude* preparations of the soluble exchange enzyme, when added to digitonin particles, show restoration of DNP-sensitivity; however, after further purification of the exchange enzyme, such "recombination" or restoration of DNP-sensitivity

TABLE 1

EFFECT OF M-FACTOR ON DNP SENSITIVITY OF ATP-ADP EXCHANGE

Exp.	Additions	ATP-ADP exchange (mμmoles ATP-C^{14})	Sensitivity to DNP (Per cent)
1.	Basic system*	200	—
	Basic system + 20 μg *N* M-factor	195	—
	Basic system + DNP	120	40
	Basic system + DNP + 5 μg *N* M-factor	100	50
	Basic system + DNP + 10 μg *N* M-factor	80	60
	Basic system + DNP + 20 μg *N* M-factor	70	65
	Basic system + DNP + 20 μg *N* M-factor (boiled)	120	
2.	Basic system*	320	—
	Basic system + DNP	250	22
	Basic system + DNP + 10 μg *N* M-factor	150	53
	Basic system + DNP + 20 μg *N* M-factor	70	78
	Basic system + DNP + 20 μg *N* M-factor + 0.002 *M* azide	320	0

* Basic test system contained 0.01 *M* ATP, 0.006 *M* ADP-C^{14} (40,000 c.p.m.), 0.001 *M* $MgCl_2$, fresh rat liver digitonin particles (64 μg *N* in exp. 1, 75 μg *N* in exp. 2) in volume of 0.5 ml. DNP was added at 5×10^{-4} *M* where shown. Incubated at 30° for 5 min. Analytical details as in rep. Ref. 2.

no longer occurs. This finding suggested that a second factor necessary for the restoration of DNP-sensitivity is removed during purification of the ATP-ADP exchange enzyme. The activity of M-factor in recombining purified ATP-ADP exchange enzyme with digitonin fragments is under examination.

Whatever the mechanism of action of M-factor, it is clear from these findings and also the experiments demonstrating the restoration of DPN-sensitivity of the soluble ATP-ADP exchange enzyme,[7] that the phosphorylating digitonin particles contain some "assemblies" of respiratory chains and energy-coupling enzymes which are incomplete in that they are lacking one or another enzyme or factor such as M-factor or the ATP-ADP exchange enzyme which may have become detached during the course of preparation of the membrane fragments. At least some of the sites which normally bind specific proteins involved in energy-coupling are still intact and able to "rebind" such proteins in a specific manner to reconstitute a functional assembly.

The relationship of the ATP-ADP exchange enzyme and M-factor to the soluble ATP-ase preparation of Pullman *et al.*[10] and the factor of Linnane and Titchener,[11] which have been shown to increase the P:O ratio of treated heart mitochondria is a point of some interest. The effect of the exchange enzyme and M-factor on the P:O ratio of digitonin fragments will be considered in detail elsewhere.

Summary.—A specific, soluble protein fraction (designated M-factor) obtained from mitochondrial extracts has been found to increase the DNP-sensitivity of

the ATP-ADP exchange reaction of oxidative phosphorylation in digitonin fragments of mitochondria. This factor is postulated to be either a "cementing" protein or an intermediate enzyme of the energy-coupling mechanism linking the terminal enzymatic step catalyzing the ATP-ADP exchange, which is DNP-insensitive, with a preceding, DNP-sensitive reaction. Preparations of M-factor also show C-factor activity, i.e., they promote contraction of glutathione-swollen mitochondria by ATP.

* Supported by grants from the National Institutes of Health, the National Science Foundation, The Nutrition Foundation, Inc., and the Whitehall Foundation.

† Senior Research Fellow, National Institutes of Health.

[1] Wadkins, C. L., *J. Biol. Chem.*, in press.

[2] Wadkins, C. L., and A. L. Lehninger, *J. Biol. Chem.*, **233**, 1589 (1958).

[3] Lehninger, A. L., C. L. Wadkins, C. Cooper, T. M. Devlin, and J. L. Gamble, Jr., *Science*, **128**, 450 (1958).

[4] Lehninger, A. L., C. L. Wadkins, and L. F. Remmert, in *Ciba Foundation Symposium on Cell Metabolism*, (London: J. and A. Churchill Ltd., 1959), p. 130.

[5] Lehninger, A. L. in "Current Topics of Interest in Biochemistry," April, 1960, *Fed. Proc.*, in press.

[6] Wadkins, C. L., *Fed. Proc.*, **18**, 346 (1959), and investigations to be published.

[7] Wadkins, C. L., and A. L. Lehninger, these PROCEEDINGS, **46**, 1576 (1960).

[8] Lehninger, A. L., and G. S. Gotterer, *J. Biol. Chem.*, **235**, PC8 (1960).

[9] Pinchot, G. B., *Biochem. Biophys., Research Commun.*, **1**, 17 (1959).

[10] Pullman, M. E., H. Penefsky, and E. Racker, *Arch. Biochem. Biophys.*, **77**, 227 (1958).

[11] Linnane, A. W., and E. B. Titchener, *Biochim. et Biophys. Acta*, **39**, 469 (1960).

---

*ON THE MUTAGENIC EFFECT OF ALKYLATING AGENTS**

BY EKKEHARD BAUTZ AND ERNST FREESE

DEPARTMENT OF GENETICS, UNIVERSITY OF WISCONSIN

Communicated by Sewall Wright, October 17, 1960

Many alkylating agents have been found to exert a mutagenic effect on a great variety of organisms.[1] These agents vary in their composition and structure from simple to very complex molecules, which will be classified here in two main groups, the mono- and polyfunctional agents.

Although, for the most part, polyfunctional agents have been used for mutation and cancer therapeutic studies investigations for a molecular explanation of mutation induction with monofunctional agents are more promising, since their reactions are more accessible to chemical analysis. In such investigations it has been found already that methyl groups attack guanine and adenine on their 7-N-position,[2] and also adenine on its 1- and 3-position,[3] whereas ethylation has been found to esterify primary phosphate groups in DNA.[2, 4]

One year ago, it was reported that ethylmethanesulfate causes high mutation rates in T4 phages.[5] Therefore, investigation of the mechanism of chemically induced mutations by alkylating agents on bacteriophage T4 seemed to us most promising since the rII region of this phage is the most extensively analyzed genetic system[6] and because of the experiences acquired in our laboratories during studies on reversion induction with nitrous acid.[7]

This paper is concerned with inactivation and with forward and reverse mutation studies on T4 phage by the action of methylating, ethylating, and propylating agents. The mutagenic specificity of ethylethanesulfonate was determined, and, in agreement with the biological results, it will be shown chemically that the main mutagenic effect of ethylation is probably due to the elimination of 7-ethylguanine from DNA.

Materials and Methods.—Phages: T4 wild type (Benzer) and rII-type mutants derived from it. *Bacteria:* B = *E. coli B* (American); K = K 12 (λ). *MMS*, *EES*, and *PPS* were prepared from the corresponding alkyliodides and silver sulfite according to the procedure of Kurbatow[6] and purified by distillation. (*MMS* = methylmethanesulfonate, *EES* = ethylethanesulfonate, *PPS* = propylpropanesulfonate.)

Inactivation of T4 phage: Various amounts of the three alkylating agents were added to 10 cc 0.5 molar sodium acetate buffer, pH 6.6. The mixtures were shaken vigorously, and after 2 min, 0.1-ml samples of T4 wild type phages were added. After various times of treatment at 37°C, 0.1-ml aliquots were diluted out into 10 ml nutrient broth containing 0.2 ml 50 per cent sodium thiosulfate, incubated for 24 hours at 37°C, and plated on bacteria B. In general, the procedure described by Loveless[5] was followed.

Mutation rates were measured, using the same conditions.

Reversion studies: The phage stocks listed in Table 1 were tested by treating the phages with 0.11 M EES in 0.5 M sodium acetate buffer, pH 6.6, at 37° for 4, 8, and 12 minutes followed by incubation for 24 hours at 37°C and were plated at a concentration of about 5×10^7 phage particles on each plate together with 5×10^8 bacteria K. This gave a multiplicity of plating 0.1, which was sufficient to show no significant amounts of multiplicity reactivation.[7] The induction of reverse mutations was tested also by preabsorption of the treated phages on bacteria B which had been killed by UV to about 10^{-3} survivals. The phages were added with a multiplicity of approximately 0.1, incubated for 8 min, and then plated on bacteria K.

Chemical analysis: Reactions on deoxyguanosine and *DNA* were performed in 1.0 M sodium acetate buffer, pH 6.6, at 37°C for 24 hours with 0.13 M dimethyl- and diethylsulfate. Using 10 mg deoxyguanosine in 10 ml buffer, the reaction mixture was applied at pH 10 to a Dowex 1 formate form anion exchange column. A fraction collector (Gilson Medical Electronics) with UV recording system at 254 mμ was employed to detect the absorbed products after elution. Spectra of the absorbing products were taken in a Zeiss spectrophotometer and compared with spectra of 7-methyladenine and 7-ethylguanine (kindly supplied by Dr. Hutchins of the Wellcome Research Laboratories). For the reaction of diethylsulfate with *DNA*, 0.5 gm of highly polymerized Herring sperm *DNA* (Sigma Chem. Co.) was suspended in 50 ml 1 M sodium acetate buffer, pH 6.6, and was dialyzed twice at 5°C against 1 liter of 1 M sodium acetate buffer for 24 hours until no further absorbing material could be detected in the dialyzate. To the *DNA* 0.8 ml of diethylsulfate was added, and the mixture was allowed to react for 24 hours at 37°C. The reaction mixture was then dialyzed against 500 cc 0.01 M saline for 24 hours at 37°C to yield approximately 90 per cent of the hydrolyzed purines in the dialyzate. To the dialyzate 0.2 gm of sodium thiosulfate was added to destroy a possible excess of unreacted diethylsulfate, and the dialyzate was concentrated to about 20 ml by evaporation at 30°C *in vacuo*. The pH was raised to 10 with ammonia and this solution was applied to the same column as described above.

Results.—Inactivation of Phage T4 was measured after treatment with *MMS*, *EES*, and *PPS* followed by incubation at 37°C for 24 hours in the presence of 1% sodium thiosulfate. A plot of the logarithm of phage survivors with respect to time of treatment usually does not give a straight line. At high concentrations of the agent, the negative slope increases, as already observed by Loveless[5] with similar alkylating agents, but at low concentrations, the negative slope decreases.

Both forward and reverse mutation rates are much larger with *EES* than with the other two agents (Figs. 1 and 2). It was found that the mutagen *EES* gave a 7- to 8-fold increase in the forward mutation frequency ($r^+ \rightarrow r$) over the spontaneous

level, at 1 per cent survivals, whereas *MMS* and *PPS* showed only a slight increase. Similarly the induction of reverse mutation by *EES* shows a 30-fold increase over the spontaneous level compared with the corresponding induction values of 1.6 for *MMS* and 2.4 for *PPS*. Absolute induction rates, the probability of reversion induction per lethal hit and phage—of 0.008 for *MMS*, 0.165 for *EES*, and 0.012 for *PPS* were calculated by a method described elsewhere.[6]

In another *EES* inactivation experiment on T4 wild type phage, using different times of incubation before plating, it could be shown (Fig. 3) that the inactivation

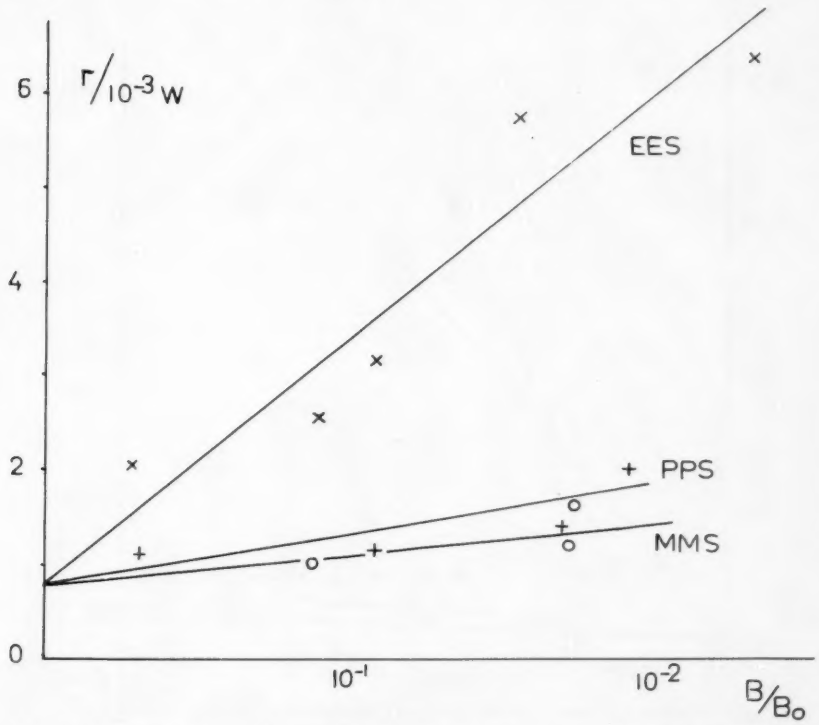


FIG. 1.—*Mutation rates.* r-/plus mottled per 1000 wild type plaques. MMS = 0.04*M*, PPS = 0.07*M*, EES = 0.11*M*.

slope increases with increasing time of incubation after treatment, whereas a control sample of phages without *EES* treatment showed no inactivation under these conditions. This gives strong evidence that the *EES* already attached to the phage causes the further inactivation upon incubation. It shows also that dilution into 1 per cent sodium thiosulfate stops further alkylation, since all slopes can be extrapolated to about 1. Plating for mutation rates with these incubation times was carried out in the same manner as described above. Plating without incubation gave about half of the number of clear r-/plus mottled plaques given by plating after 24 hours (Fig. 1). Moreover, mostly mottled plaques were ob-

served for this direct plating after treatment while furthur incubation enhanced the frequency of both *r* and mottled plaques.

Reversion studies: EES, the most efficient of the three agents, was chosen for reversion tests on two classes of rII-type mutants—those induced with 5-bromouracil (N mutants) and those with 2-aminopurine (AP mutants). The results are given in Table 1. The mutants seem to fall into two distinct groups: those capable of being reverted by *EES* (AP mutants) and those showing little or no response to the agent (N mutants). By plating the inactivated phages directly

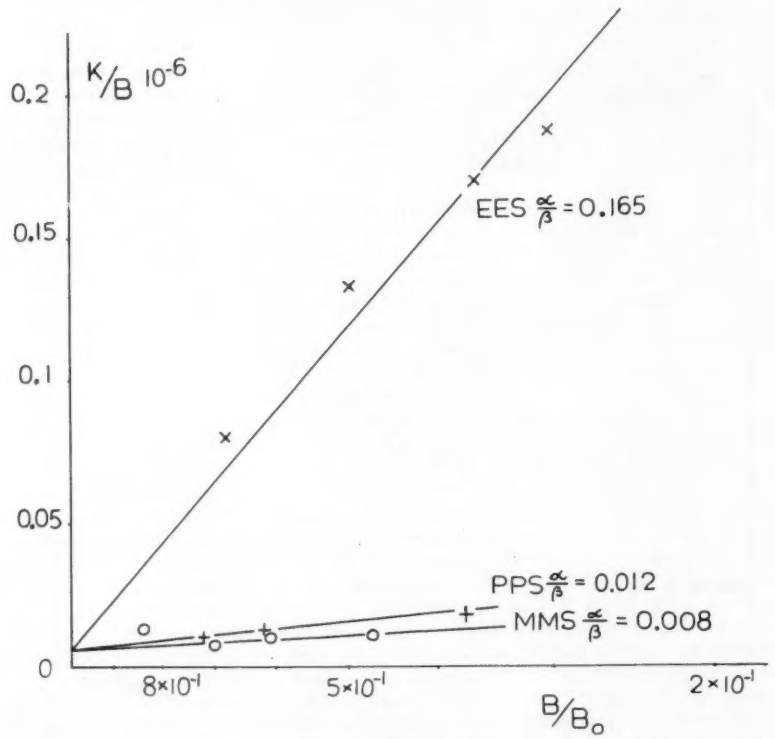


FIG. 2.—*Reversion rates.* Back mutated wild type plaques per 10^6 viable r-mutants of AP 156. MMS = 0.04 *M*, PPS = 0.07 *M*, EES = 0.11 *M*. The value α/β = the increase of revertants per viable phage over the spontaneous level at ln $B/B_0 = 1$.

on bacteria *K*, for the AP mutants the values of the total mutation induction, α/β, vary between 4.6×10^{-6} for AP 12 and 0.065×10^{-6} for AP 70. All the N mutants except N 29 and N 19 showed no detectable rate of induction, which is indicated by $\alpha/\beta < 0.01$. N 29 happens to be a very reactive mutant and also shows the highest induction with nitrous acid among the N mutants,[7] and is strongly inducible by AP.[9]

Each of five mutant strains of the N and the AP type was tested with preadsorption on bacteria *B*, and the results are given in the last row of Table 1. The AP

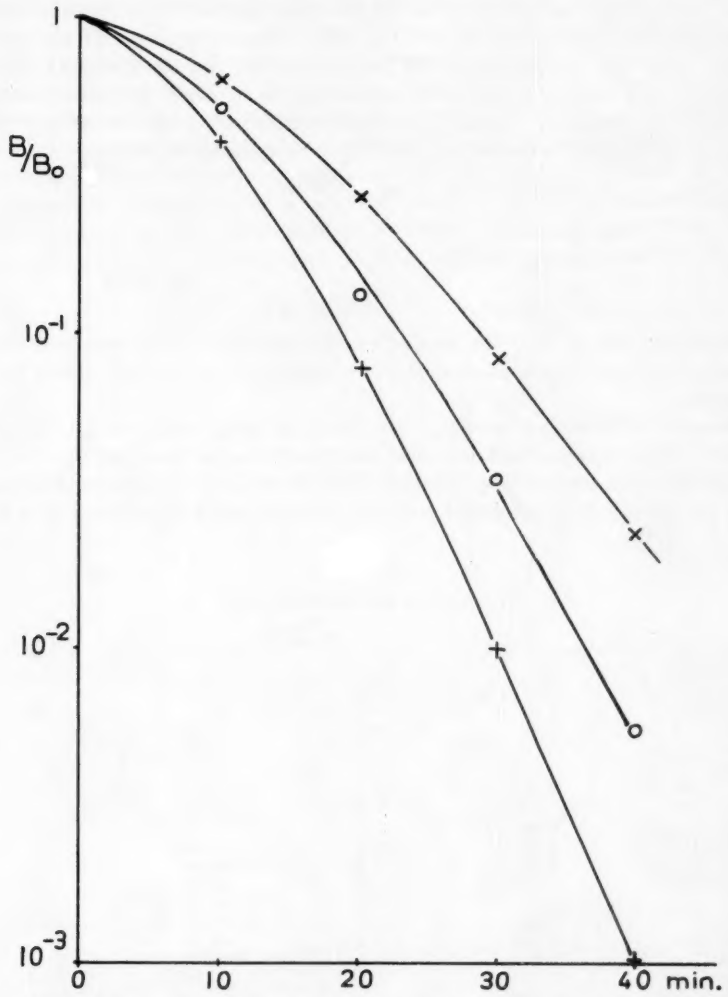


FIG. 3.—*Inactivation curves* of T4 wild type phages treated with 0.11 *M* EES, followed by incubation. × = 0 hours' incubation; 0 = 12 hours' incubation; + = 24 hours' incubation.

mutants show an even higher increase in the reversion rates, whereas the N mutants show no effect. Although the increase of reversion rates for the AP mutants on the average is 10 times as high as without preadsorption, the accuracy of these values is much lower, because the increase of the spontaneous background is also 10 to 100 times higher than without preadsorption.

Chemical Analysis.—Alkylation of deoxyguanosine: Samples of 5 mg each of deoxyguanosine were incubated with 0.15 *M* dimethyl- and diethylsulfate for 24 hours at 37° at pH 6.6 and applied to the column. The deoxyguanosine alkylated

at the 7-N-position was not absorbed by the resin, because of its positive charge, and it could be collected in the first fractions. Elution with 0.2 *M* ammonium formate buffer pH 9.5 separated the 7-alkylguanine from the unreacted deoxyguanosine. UV analysis at pH 9.5 showed a λ max at 283 mμ for both the 7-methyl- and the 7-ethylguanine. Assuming a molar extinction coefficient of $8 \times 10^{+3}$ at λ max for all guanine derivatives, the following amounts were detected:

7-ethyldeoxyguanosine	0.08 mg	ratio 1:4
7-ethylguanine	0.33 mg	
7-methyldeoxyguanosine	0.7 mg	ratio 2.5:1
7-methylguanine	0.28 mg	

Comparing these ratios with each other, the hydrolysis of the ethylated deoxyguanosine appears 5 times as efficient as the hydrolysis of the methylated deoxyguanosine.

Estimation of hydrolyzed purines from DNA: A large sample of 0.5 *DNA* was treated with diethylsulfate as described above and was dialyzed, and the dialyzate was applied to the ion-exchange column. Elution with pH 9.5 ammonium formate buffer produced first nonabsorbed positively charged material, followed by a large

TABLE 1

INDUCTION OF REVERSIONS BY *EES*

Mutant	K_0/B_0 in 10^{-6}	Without Preadsorption I	Without Preadsorption α/β in 10^{-6}	With Preadsorption α'/β in 10^{-6}
AP 12	0.85	5.4	4.6	..
AP 41	0.37	6.2	2.3	..
AP 275	1.07	1.5	1.5	18
AP 114	0.49	2.8	1.35	..
AP 72	0.76	1.6	1.24	20
AP 83	0.006	43.3	0.26	8.5
AP 156	0.005	31.1	0.165	0.9
AP 70	0.028	2.3	0.065	4.0
N 29	0.04	1.7	0.03	<0.1
N 19	0.07	1.2	0.016	..
N 24	0.32	1.0	<0.01	..
AP 61	0.18	1.0	<0.01	<0.1
N 31	0.01	1.0	<0.01	..
N 12	0.03	1.0	<0.01	<1
N 101	0.002	1.0	<0.01	..
N 17	0.02	1.0	<0.01	..
N 7	0.03	1.0	<0.01	<0.1
N 21	0.006		<0.01	<0.1
r 114	0.20	1.0	<0.01	..
r 131	0.43	1.0	<0.01	..

For each mutant, the spontaneous reversion rates K_0/B_0, the induction factor $I = K/B : K_0/B_0$, and the absolute rates of reversion induction α/β are given per lethal hit for direct plating on bacteria *K*. "*K*" = titer on bacteria *K*, "*B*" = titer on bacteria *B*. The reversion rates α/β obtained after preadsorption of the phages on bacteria *B* are given in the last row of Table 1. $\alpha/\beta <$ means no detectable amount of induction. The mutant strain AP 61 was previously found to belong to the N-mutant group.[7,9]

peak, identified as 7-ethylguanine. A peak of 7-ethyladenine, normally appearing among the first fractions after adjustment of the eluant to pH 8.5, could not be detected. Calculation of the total amount of eluted 7-ethylguanine gave an optical density at pH 9.5 of 0.19 per 100 ml. Compared with the molar extinction coefficient of $8 \times 10^{+3}$, 2.2 micromoles = 0.4 mg of 7-ethylguanine were recovered. Starting with 0.5 gm *DNA*, which contains about 60 mg guanine, the yield was

about 0.6 per cent depurination during 24 hours treatment, followed by 24 hours incubation while dialyzing. The smallest amount of 7-ethyladenine that could have been detected is 0.4 micromoles. Thus we conclude that the formation of 7-ethyladenine was at least 50 times less frequent than that of 7-ethylguanine.

Discussion.—It has been found[9] that bromouracil-induced mutants show higher induction of reverse mutations by 2-aminopurine than by bromodeoxyuridine and vice versa. These observations suggested that most mutants induced by one of the two agents arose by a change of an adenine-thymine (A-T) to a guanine-cytosine (G-C) base pair and that the mutants induced by the other agent arose by a change of a G-C to an A-T base pair. Nitrous acid was hoped to be a suitable agent

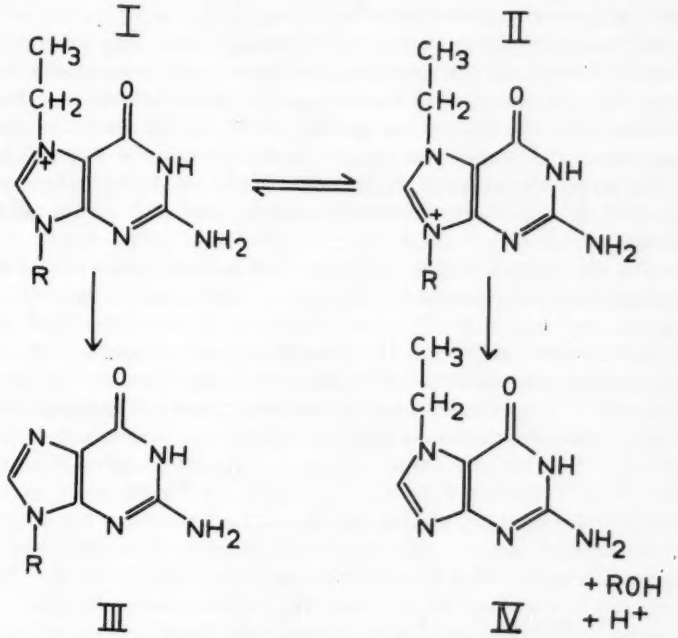


FIG. 4.—*Proposed mechanism* of depurination by ethylation. I and II: mesomeric states of 7-ethyldeoxyguanosine. IV: removal of R- caused by state II. R- = deoxyribosyl-.

to decide by reversion tests which one of the two base pairs was changed in the cases of AP mutants and of N mutants; but the small difference in reaction velocity of nitrous acid with the two base pairs[10, 11] did not allow a definite measurement of the specificity of its mutagenic action.

Ethylation, on the other hand, causes a rather specific mutagenic effect, as was found by Krieg,[12] who, using T4 phages, demonstrated that mutants produced by the action of ethylmethanesulfonate (*EMS*) cannot be induced to revert by the same agent. The results in Table 1 indicate that the specificity of *EES* turns out to be sufficient to differentiate between the *AP* mutant having the lowest reversion rate by *EES* and the highest revertible N mutant. The result of these reversion

studies demonstrates a high specificity of *EES* which probably can be used to analyze the base-pair change which has been induced by other mutagens. In order to obtain quantitative chemical evidence for the biological specificity of *EES*, we had to find out first which one of the different reactions of alkylating agents on *DNA* could be made responsible for the induction of mutation.

Reiner and Zamenhof,[2] treating transforming *DNA* with dimethylsulfate, have shown that 7-methylguanine and 7-methyladenine were two of several products obtained after hydrolysis. Purines were found to be much more reactive than the pyrimidines. Using diethylsulfate, they found only esterification with primary phosphate groups, which also was reported by Alexander and Stacey.[3] Recently, Brooks and Lawley[4] reported methylation of adenylic acid to give, in addition, 1-methyl- and 3-methyladenine after hydrolysis.

The reaction of ethylating agents with primary phosphate groups in *DNA* seems not to be responsible for the induction of point mutations, which we consider here, since the subsequent effect could be only a break in the phosphate-sugar backbone similar to the decay of incorporated P^{32} which has not yet been found to cause mutations. Moreover, this reaction would not give rise to single-base-pair changes but larger alterations in the genom. Hence, we conclude that one of the reactions with the *DNA* bases themselves must be responsible for the induction of point mutations.

Regarding the reaction on the 3-N-position of adenine, alkylation would yield an uncharged 3-alkyl-6-iminopurine as product, which could behave like guanine when pairing and simultaneously cause a base-pair change in the *DNA* molecule. The cause of mutation might thus be answered, but not the question why methylation causes much less mutation than does ethylation, because the reaction of methyl groups with the ring nitrogens in purines is known to go much faster than ethylation. These contradictions made us assume that there must be a further reaction within the alkylated *DNA*. In our experiments, ethylation of *DNA* for 24 hours at 37°C, followed by further incubation for 24 hours at the same temperature and neutral pH, yielded 0.6 per cent 7-ethylguanine but no detectable amount of ethylated adenine. Methylation, in contrast, is less effective in breaking the N-glycosidic bond and also is less mutagenic (see Figs. 1 and 2). These observations can be explained by the following theoretical considerations.

Ethylation at the 7-N-position of guanine uses the free electron pair of the 7-N-atom of the imidazole ring to bind the ethyl group, causing a full positive charge as shown in Figure 4, structure I. This structure stays in resonance with structure II, yielding a resonance hybrid. The positive charge, distributed to both N-atoms of the imidazole ring, greatly weakens both the 7-N-ethyl and the 9-N-sugar bonds, so that hydrolysis takes place even at room temperature and neutral pH. Figure 4 shows the following simplified and idealized mechanism. The imidazole ring, ethylated on its 7-N-atom, has gained a positive charge, which is distributed over the ring, yielding to the two extremal states I and II. State I enables the removal of the $C_2H_5^+$ group from the guanine nucleotide, yielding back the unchanged *DNA*. Structure II, however, causes the eventual hydrolysis of the N-glycosidic bond, thus removing guanine and changing the genetic properties of its *DNA*. Methylation also induces a positive charge in the imidazole ring, but the methyl group is able to carry a part of the charge because of its

electron-inducing capacity; i.e., methyl groups are able to participate in the resonance of the imidazole ring, which simultaneously causes less positive charge at the 9-N-atom and also less hydrolysis of the N-sugar bond.

Some further biological results, which are given in Figure 3, support the theory above. From the curves, it can be seen that the titer of the inactivated phages shows a further decrease after incubation at 12 and 24 hours at 37°C, depending on the number of ethyl groups the phages originally had received. This behavior would be expected, since, during incubation, more and more ethylated guanines get removed from *DNA*.

Once a *DNA* molecule, missing a guanine, duplicates, one would expect the incorporation of either a purine or pyrimidine into the new complementary strand, giving rise to both transversions and transitions.[14] It was observed that depurination caused by high temperature and by low pH produces mutations[13, 14] and that these mutations have been produced mainly by transitions.[14]

From this point of view, we can say that the depurination by *EES* causes also mutation by substitution of adenine for the guanine lost under the action of ethyl groups. Nevertheless, there might be also a certain amount of mutations due to transversions, which could not have been detected in our reversion studies.

If we compare the high inducibility of the AP mutants and the inert behavior of the N mutants with the chemical results, we have evidence for the following conclusions: Following the reaction of ethylating agents on *DNA*.

1. Liberation of 7-ethylguanine occurs at least 50 times more frequently than of 7-ethyladenine.

2. From our experimental results and the theoretical considerations, we can strongly suggest that the removal of guanine has to be the main cause of mutation induction by ethylethanesulfonate.

3. The tested mutants induced by the two base analogues, 2-aminopurine and 5-bromouracil, arose by the following base pair changes:

$$\begin{matrix} A & G \\ & \rightarrow \\ T & C \end{matrix}$$ for most mutants induced by 2-aminopurine

$$\begin{matrix} G & A \\ & \rightarrow \\ C & T \end{matrix}$$ for most mutants induced by 5-bromouracil

This latter conclusion is made with the assumption that our reverse mutations are back mutations to the original T4 wild type, which will be discussed in a subsequent paper.[15]

* Paper No. 804 from the Department of Genetics, University of Wisconsin. This study was supported in part by a grant from the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation and by grants of the U.S. Public Health Service (Grant RG-7119) and the Damon Runyon Memorial Fund for Cancer Research.

[1] Westergaard, M., *Experientia*, **13**, 224 (1957).
[2] Reiner, B., and S. Zamenhof, *J. Biol. Chem.*, **228**, 475 (1957).
[3] Alexander, P., and K. A. Stacey, *Ann. N. Y. Acad. Sci.*, **68**, 1225 (1958).
[4] Brooks, P., and P. D. Lawley, *J. Chem. Soc.*, **112**, 539 (1960).
[5] Loveless, A., *Proc. Roy. Soc.*, **150B**, 423, 486, 497 (1959).
[6] Benzer, S., these PROCEEDINGS, **41**, 344–354 (1955).

[7] Bautz Freese, E., and E. Freese, *Virology*, in press.
[8] Kurbatow, A., *Liebigs Ann. Chem.*, **173**, 7 (1874).
[9] Freese, E., these PROCEEDINGS, **45**, 622 (1959).
[10] Schuster, H., *Biochem. and Biophys. Res. Comm.*, **2**, 320 (1960).
[11] Vielmetter, W., and H. Schuster, *Biochem. and Biophys. Res. Comm.*, **2**, 324 (1960).
[12] Krieg, D. R. (personal communication).
[13] Greer, S., and S. Zamenhof, *Fed. Proc.*, **18**, 938 (1959).
[14] Freese, E., *Brookhaven Symp. Quant. Biol.*, **12**, 63 (1959).
[15] Freese, E., E. Bautz, and E. Bautz Freese (in prep.).

---

*SOME SINGULAR MIXED PROBLEMS**

BY ROBERT CARROLL

DEPARTMENT OF MATHEMATICS, RUTGERS, THE STATE UNIVERSITY

Communicated by Einar Hille, October 14, 1960

1. *Introduction.*—In this note we shall consider the differential operator $L^k = \partial^2/\partial t^2 + [k/t + \alpha(t)]\partial/\partial t + \gamma(t)$, where k is complex with $\mathrm{Re}\,(k) > 0$, $\alpha \epsilon C'([0, b])$, and $\gamma \epsilon C^0([0, b])$. (The case $k = 0$ may be treated similarly.) $C^m([0, b])$ is the space of m-times continuously differentiable functions on $[0, b]$ and the number $b < \infty$ is arbitrary but fixed. Let $\mathfrak{H}$ be a separable Hilbert space, and Λ an arbitrary self-adjoint operator, semibounded below, with domain $D(\Lambda)$ (dense in $\mathfrak{H}$). We will consider the problem

$$(L^k + \Lambda)\omega^k(t) = 0;\ \omega^k(\tau) = T \epsilon D(\Lambda);\ \partial/\partial t \omega^k(\tau) = 0, \tag{1}$$

for $t \to \omega^k(t) \epsilon\, \mathcal{E}^2(\mathfrak{H})$, $t \to \omega^k(t) \epsilon\, \mathcal{E}^0(D(\Lambda))$, and $0 \leq \tau \leq t \leq b$. $\mathcal{E}^m(\mathfrak{H})$ is the space of m-times continuously differentiable functions with values in $\mathfrak{H}$, and we assume here that $D(\Lambda)$ has the topology of the graph, under which it is a Hilbert space. Since we are assuming of $\gamma(t)$ only that it be continuous, it is clear that the case of semi-bounded Λ is equivalent to that of Λ positive, which we shall suppose from now on.

Problems similar to (1) have been considered by J. L. Lions[2, 3] and the author,[4] while the case $\alpha = \gamma \equiv 0$ goes back to the Euler-Poisson-Darboux (EPD) equation which has been treated in particular by A. Weinstein[5] in a long series of papers. More abstractly the EPD equation is discussed in refs. 1, 6, and 7. In the present note we shall apply a method used in ref. 4 (where Λ is a differential operator with constant coefficients and $\mathfrak{H}$ is replaced by $\mathcal{S}'$ etc.) to the problem (1). This method is based on techniques of L. Schwartz.[8] Our results refine in some respects, for $\mathrm{Re}\,(k) > 0$, those of Lions.[2, 3]

Using now the von Neumann spectral decomposition theorem (see for example ref. 9), we know there is a measure ν, a ν-measurable family of Hilbert spaces $\zeta \to H(\zeta)$, and an isometric isomorphism θ: $\mathfrak{H} \to H = \int^{\oplus} H(\zeta) d\nu(\zeta)$, such that Λ is diagonalized, with $T \epsilon D(\Lambda)$ if and only if $\int_0^\infty \zeta^2 \|\theta T\|^2_{H(\zeta)} d\nu(\zeta) < \infty$. The problem (1) is then equivalent to seeking $\theta\omega^k(t, \zeta) \epsilon \theta D(\Lambda)$ such that

$$(L^k + \zeta)\theta\omega^k(t, \zeta) = 0;\ \theta\omega^k(\tau, \zeta) = \theta T \epsilon \theta D(\Lambda);\ \frac{\partial}{\partial t}\theta\omega^k(\tau, \zeta) = 0, \tag{2}$$

with $t \to \theta\omega^k(t, \zeta) \epsilon\, \mathcal{E}^2(H)$ and $t \to \theta\omega^k(t, \zeta) \epsilon\, \mathcal{E}^0(\theta D(\Lambda))$.

2. *Existence.*—From the results of ref. 4, we know there exists a function $Z^k(t, \tau, \zeta)$ $(0 \leq \tau \leq t \leq b)$ with the following properties ($Z_t(t, \tau, \zeta) = \partial/\partial t Z(t, \tau, \zeta)$ etc.): (i) $(L^k + \zeta)Z^k(t, \tau, \zeta) = 0$; $Z^k(\tau, \tau, \zeta) = 1$; $\partial/\partial t Z^k(\tau, \tau, \zeta) = 0$; (ii) Z^k, $Z_t{}^k$ and $Y^k = (\gamma(\tau) + \zeta)^{-1} Z^k{}_\tau$ are continuous in all variables (t, τ, ζ), $0 \leq \tau \leq t \leq b$, and, with $Z_{tt}{}^k$, are analytic in ζ for (t, τ) fixed; (iii) $Z_{tt}{}^k$ is continuous in (t, ζ) for τ fixed; (iv) the function $Y^k(t, \tau, \zeta)$ satisfies the equation $Y^k{}_\tau = -Z^k + \left[\frac{k}{\tau} + \alpha(\tau)\right] Y^k$, with $Y^k(\tau, \tau, \zeta) = 0$ and $Y^k(t, 0, \zeta) = 0$. Moreover, there are constants c_i independent of τ, $t (0 \leq \tau \leq t \leq b)$ and ζ for ζ real $(\zeta \geq 0)$ such that

$$|Z^k(t, \tau, \zeta)| \leq c_1; \quad |Z_t{}^k(t, \tau, \zeta)| \leq c_2 + c_3 \zeta^{1/2}; \quad |Z_{tt}{}^k(t, \tau, \zeta)| \leq c_4 + c_5 \zeta; \quad |Y^k(t, \tau, \zeta)| \leq c_6. \tag{3}$$

We set now $\theta\omega^k(t, \zeta) = Z^k(t, \tau, \zeta)\theta T$ and using the Lebesgue bounded convergence theorem it may be shown that $t \to \theta\omega^k \epsilon\, \mathcal{E}^0(\theta D(\Lambda))$ and $t \to \theta\omega^k \epsilon\, \mathcal{E}^2(H)$ with $\theta\omega_t{}^k = Z_t{}^k \theta T$ and $\theta\omega_{tt}{}^k = Z_{tt}{}^k \theta T$. Thus,

THEOREM 1. *Under the assumptions* $T \epsilon D(\Lambda)$, $\alpha \epsilon C'$, $\gamma \epsilon C^0$ *and* $\mathrm{Re}(k) > 0$ *the function* $\omega^k(t, \tau) = \theta^{-1}\{Z^k(t, \tau, \zeta)\theta T\}$ *is a solution of the Cauchy problem* (1).

3. *Uniqueness.*—We will first give a uniqueness theorem modeled after that of ref. 4. Let $\mathfrak{L}_s(E, F)$ be the space of continuous linear applications $E \to F$ with the topology of simple convergence; we write also $\mathfrak{L}_s(H, H) = \mathfrak{L}(H)$. When E is *tonnelé* we know[10] that the separately continuous application $(\beta, e) \to \beta e$: $\mathfrak{L}_s(E, F) \times E \to F$ is continuous when β remains in a bounded set B in $\mathfrak{L}_s(E, F)$. Now from the above we have $\xi \to Y^k(t, \xi, \zeta) \epsilon\, \mathcal{E}^0(\mathfrak{L}(H))$ (and $\mathcal{E}^0(\mathfrak{L}(\theta D(\Lambda)))$ and $Y^k \epsilon\, \mathfrak{L}(H)$ remains bounded for $0 \leq \xi \leq t$ (assume t fixed here). The same holds for $\xi \to Z^k(t, \xi, \zeta)$ and hence for $\xi \to \xi Y_\xi{}^k(t, \xi, \zeta)$. Moreover, $\xi Y_\xi{}^k \to 0$ as $\xi \to 0$ and, for $\theta S \epsilon H$, $\xi \to Y_\xi{}^k(t, \xi, \zeta)\theta S \epsilon L^1(H)$ (for $\xi > 0$, $\xi \to Y^k \epsilon\, \mathcal{E}'(\mathfrak{L}(H))$ or $\mathcal{E}'(\mathfrak{L}(\theta D(\Lambda)))$). Also, considering Z^k and $Z_\xi{}^k \epsilon\, \mathfrak{L}_s(\theta D(\Lambda), H)$, it may be shown that $\xi \to Z^k(t, \xi, \zeta) \epsilon\, \mathcal{E}'(\mathfrak{L}_s(\theta D(\Lambda), H))$. The Lebesgue bounded convergence theorem is again useful in demonstrating these facts.

We now assume ω^k is a solution of problem (1). We rewrite (2) with t replaced by ξ, considering this as an equation in H, and "multiply" by $Y^k(t, \xi, \zeta)$ considered as an operator. The above comments show that the terms $\xi \to Y_\xi{}^k(t, \xi, \zeta)\theta\omega_\xi{}^k(\xi, \tau, \zeta) = \xi Y_\xi{}^k \frac{1}{\xi} \theta\omega_\xi{}^k$ etc. are continuous functions of ξ with values in H, and may be used to justify the integration by parts which follows.

$$\int_\tau^t \left\{ Y^k(t, \xi, \zeta)\theta\omega_{\xi\xi}{}^k(\xi, \tau, \zeta) + \left(\frac{k}{\xi} + \alpha\right) Y^k \theta\omega_\xi{}^k + (\zeta + \gamma) Y^k \theta\omega^k \right\} d\xi$$
$$= \theta\omega^k(t, \tau, \zeta) - Z^k(t, \tau, \zeta)\theta T + \int_\tau^t \left\{ - Y_\xi{}^k + \left(\frac{k}{\xi} + \alpha\right) Y^k - Z^k \right\} \theta\omega_\xi{}^k d\xi = 0. \tag{4}$$

We have used the boundary conditions as well. To show that the last integral vanishes in (4), one may take its scalar product with an arbitrary $\theta S \epsilon H$ and then decompose the terms. This proves

THEOREM 2. *The solution of problem* (1) *given by theorem* 1 *is unique for* $T\epsilon D(\Lambda)$ $\alpha\epsilon C'$, $\gamma\epsilon C^0$ *and* Re $(k) > 0$.

Combining these theorems with (ii) and (3) above, we see that $\theta\omega^k = Z^k(t, \tau, \zeta)\theta T$ depends continuously in $\theta D(\Lambda)$ on t, τ and θT. In the terminology of ref. 8, we have

THEOREM 3. *The problem* (1) *is uniformly well posed in* $\mathfrak{H}$.

4. *Variations on Uniqueness.*—We will sketch here another theorem of uniqueness, under slightly different hypotheses, based on results of J. L. Lions. Assume $\mathfrak{H}$ is an arbitrary Hilbert space, $\alpha\epsilon C^0$, $\gamma\epsilon C^0$, Re $(k) \geq 0$, $t \to \omega^k(t)\epsilon\, \mathcal{E}'$ $(D(\Lambda), t \to \omega^k(t)\epsilon\, \mathcal{E}^2$ $(\mathfrak{H})$ and $(L^k + \Lambda)\omega^k = 0$ with $\omega^k(\tau) = \omega_t{}^k(\tau) = 0$, $\tau \geq 0$. By changing $\gamma\epsilon C^0$ if necessary, we may assume also here that $(\Lambda h \mid h) \geq \|h\|^2{}_{\mathfrak{H}}$. Take now the scalar product $((L^k + \Lambda)\omega^k \mid \omega_t{}^k)_{\mathfrak{H}}$ and add to this its complex conjugate. Integrating and using the boundary conditions, we have

$$\|\omega_t{}^k(t)\|^2{}_{\mathfrak{H}} + \|\omega^k(t)\|^2{}_{\mathfrak{H}} \leq \|\omega_t{}^k(t)\|^2{}_{\mathfrak{H}} + (\Lambda\omega^k \mid \omega^k)_{\mathfrak{H}} + \int_\tau^t \frac{2 \text{ Re } (k)}{\xi} \|\omega_\xi{}^k(\xi)\|^2{}_{\mathfrak{H}} d\xi =$$

$$-\int_\tau^t 2 \text{ Re } (\alpha)\|\omega_\xi{}^k\|^2 d\xi - \int_\tau^t 2 \text{ Re } [\gamma(\omega^k \mid \omega_\xi{}^k)]d\xi \leq c \int_\tau^t \left\{\|\omega_\xi{}^k\|^2{}_{\mathfrak{H}} + \|\omega^k\|^2{}_{\mathfrak{H}}\right\} d\xi. \tag{5}$$

where $c_1 = 2 \sup |\text{Re } (\alpha)|$, $c_2 = \sup |\gamma|$ on $[0, b]$ and $c = c_1 + c_2$. However, this implies that $\|\omega_t{}^k\| = \|\omega^k\| = 0$ and we may state

THEOREM 4. *Problem* (1) *has a unique solution for* $\alpha\epsilon C^0$, $\gamma\epsilon C^0$, Re $(k) \geq 0$ and $\mathfrak{H}$ *an arbitrary Hilbert space, if it is assumed that* $t \to \omega^k(t)\epsilon\, \mathcal{E}^2$ $(\mathfrak{H})$ *and* $t \to \omega^k(t)\epsilon\, \mathcal{E}'$ $(D(\Lambda))$.

Remark 1: Theorem 4 is stronger than theorem 2 except that it requires in particular that $t \to \omega_t{}^k(t) : R \to D(\Lambda)$ be continuous. We note by (3) that the solution given by theorem 1 does not seem to have this property unless $T\epsilon D(\Lambda^{1/2})$.

Remark 2: Using a method different from that of ref. 4, it may be shown that $\alpha\epsilon C^0$ will suffice in sections 2 and 3; we will treat this as well as more general types of singularity in a paper in preparation.

* This work was supported by the National Science Foundation under a fellowship grant.

[1] Lions, J. L., *Bull. Soc. Math. France*, **84**, 9–95 (1956).
[2] Lions, J. L., *Comptes Rendus Acad. Sci.*, **246**, 208–210 (1958).
[3] Lions, J. L., *Rend. Sem. Mat. Fis. Milano*, **28**, 3–16 (1959).
[4] Carroll, R., *Comptes Rendus Acad. Sci.*, **251**, 498–500 (1960).
[5] Weinstein, A., *Summa Brasil. Math.*, **3**, 125–147 (1955).
[6] Krasnov, M. L., *Doklady Akad. Nauk USSR*, **107**, 6, 789–792 (1956).
[7] Carroll, R., *Annali di Mat.*, article to appear.
[8] Schwartz, L., *Annales Inst. Fourier*, **2**, 19–49 (1950).
[9] Dixmier, J., *Les algèbres d'opérateurs dans l'espace Hilbertien*, (Paris, 1957).
[10] Bourbaki, N., *Espaces vectoriels topologiques* (Paris, 1955).

OPTIMAL GAMBLING SYSTEMS

By Lester E. Dubins and Leonard J. Savage

UNIVERSITY OF CALIFORNIA AT BERKELEY AND UNIVERSITY OF MICHIGAN

Communicated by J. L. Doob, October 19, 1960

Until recently, the mathematics of optimal gambling has been primarily concerned with the formulation and proof of variants of the following fact. The expected terminal fortune of a gambler, whose fortune is always nonnegative, engaged in any succession of gambles that are fair or unfair to him cannot be larger than his initial fortune.

These results, though important, do not say how well the gambler can do in given unfair gambling situations, nor do they suggest what gambling strategy is best in his bad situation. These latter problems have their own mathematical interest, and the underlying ideas used in attacking them can be applied to establish various inequalities for wide classes of stochastic processes of theoretical, and perhaps practical, interest.

Some illustrations of our results in this area, to be published elsewhere, are here stated without proof in language that, for brevity, is somewhat informal.

Let r and w be two numbers less than 1. Suppose a gambler whose initial fortune is positive can make a sequence of bets, on any one of which he can stake any amount then in his possession. Each bet results in either a gain or a loss, where the ratio of the possible gain to the possible loss is as $1 - r$ to r, the gain occurs with probability w, and the loss occurs with probability $1 - w$.

THEOREM 1. *If $w > r$, there exists k, $0 < k < 1$, such that a gambler who stakes cy whenever his fortune is y will have his fortune converge almost certainly to $+\infty$ or 0, according as $0 < c < k$ or $c > k$. If $c = k$, the* lim sup *and* lim inf *of his fortunes will almost certainly be $+\infty$ and 0, respectively.*

Let a certain positive G be a gambler's goal. Roughly speaking, a betting strategy is *optimal* if it maximizes the probability that the gambler's fortune ever attains the goal. A gambler *plays boldly* if he always stakes as much as possible consistent with the condition that his fortune never exceed G or become negative.

THEOREM 2. *If $w < r$, bold play is optimal. Bold play is not the only optimal strategy.*

Let g be a random variable bounded below by -1 and of expectation 0—a fair lottery, so to speak—and let g_n, $n = 1, 2, \ldots$, be independent, each with the same distribution as g. For any x, $0 < x < 1$, consider any stochastic process f_n, $n = 0, 1, 2, \ldots$—the fortune of a gambler at time n—such that: (i) $f_0 = x$; (ii) $f_n \geq 0$ for all n; (iii) $f_{n+1} = f_n + s_{n+1}g_{n+1}$, where s_n—the stake at time n—is nonnegative and may depend upon $f_0, \ldots, f_n$. Then $\sigma = s_1, s_2, \ldots$ is a *strategy* and $p(\sigma, x)$ is the probability that for some n, $f_n \geq 1$. Before stating a main result, we note that well-known theorems easily imply: (iv) For all σ, $p(\sigma, x) \leq x$; (v) if g is bounded above and if its upper bound is attained with positive probability, then there is a σ such that $p(\sigma, x) = x$; (vi) if g is bounded above but its upper bound is not attained with positive probability, then for all σ and x, $p(\sigma, x) < x$ but the sup of $p(\sigma, x)$ over all σ is x.

Suppose now that g is unbounded above. It is relatively easy to exhibit a g

such that $U(x)$, the supremum of $p(\sigma,x)$ over all σ, is x. It is much more difficult to see, as Donald Ornstein was the first to do, that there exist g for which $U(x) < x$. We now know more:

THEOREM 3. *Suppose that g has mean 0 and that $|g|$ has positive mean. Then in order that $U(x) = x$ it is necessary and sufficient that the following condition hold: The limit inferior, as z approaches ∞, of $(-z\int_{g<z} g)(\int_{g<z} g^2)^{-1}$ is 0.*

This research owes much to many, both for ideas and for financial support, as will be explained in the full publication.

FIELDS OF UNIT VECTORS IN THE FOUR-SPACE OF GENERAL RELATIVITY

BY LUTHER P. EISENHART

PRINCETON UNIVERSITY

Communicated October 14, 1960

1. A previous paper[1] deals with the tangent vectors to the minimal geodesics of the four-space of general relativity V_4, which, according to Einstein, are the paths of light.[2] This means that the tensor g_{ij} in the fundamental equation

$$ds^2 = g_{ij}dx^i dx^j \tag{1}$$

is not positive definite. The right-hand member of this equation stands for the sum of terms as i and j take the values 1 to 4.

This convention is used throughout this paper, namely, that when within a term the same letter enters as a superscript and subscript, the term stands for the sum of terms as the index takes the values 1 to 4.

The present paper deals with unit vectors in the V_4, that is,

$$\lambda_{1;}{}^i\lambda_{1;i} = 1, \quad \lambda_{2;}{}^i\lambda_{2;i} = 1, \quad \lambda_{3;}{}^i\lambda_{3;i} = 1, \tag{2}$$

which are mutually orthogonal, that is,[3]

$$\lambda_{1;}{}^i\lambda_{2;i} = 0, \quad \lambda_{2;}{}^i\lambda_{3;i} = 0, \quad \lambda_{3;}{}^i\lambda_{1;i} = 0. \tag{3}$$

2. We use the null vector λ_i of the previous paper, that is,

$$\lambda_i\lambda^i = 0, \tag{4}$$

and require that the vectors $\lambda_{1;i}$, $\lambda_{2;i}$, $\lambda_{3;i}$ be orthogonal to the vector λ_i, that is,

$$\lambda_{1;i}\lambda^i = \lambda_{1;}{}^i\lambda_i = 0, \ \lambda_{2;i}\lambda^i = \lambda_{2;}{}^i\lambda_i = 0, \quad \lambda_{3;i}\lambda^i = \lambda_{3;}{}^i\lambda_i = 0. \tag{5}$$

We put

$$\lambda_{1;i,j} = \lambda_i\lambda_{1;j}, \quad \lambda_{2;i,j} = \lambda_1\lambda_{2;j}, \quad \lambda_{3;i,j} = \lambda_i\lambda_{3;j}, \tag{6}$$

where the terms on the left are the covariant derivatives of $\lambda_{1;i}$, $\lambda_{2;i}$, $\lambda_{3;i}$ with respect to x^j.[4]

This convention is used throughout this paper, namely, a component of a vector

followed by a comma and index denotes the covariant derivative of the vector with respect to x with this index.

Since a covariant derivative of g^{ik} is equal to zero,[5] it follows that

$$g^{ik}\lambda_{1;i,j} = (g^{ik}\lambda_{1;i})_{,j} = \lambda_{1;}{}^{k}{}_{,j},$$

by means of which from the first of equations (6) we have

$$\lambda_{1;}{}^{k}{}_{,j} = \lambda^{k}\lambda_{1;j}$$

which when k is replaced by i becomes

$$\lambda_{1;}{}^{i}{}_{,j} = \lambda^{i}\lambda_{1;j}. \tag{7}$$

When the same process is applied to the second and third of equation (6), the result is

$$\lambda_{2;}{}^{i}{}_{,j} = \lambda^{i}\lambda_{2;j}, \quad \lambda_{3;}{}^{i}{}_{,j} = \lambda^{i}\lambda_{3;j}. \tag{8}$$

3. Equation (12) of the previous paper is

$$\lambda_{i,j} = a\lambda_i\lambda_j + b\lambda_i\mu_j + c\mu_i\lambda_j \tag{9}$$

where a, b, c are constants, and the vector μ_i is such that

$$\mu_i\lambda^i = 0, \quad \mu_i\mu^i = 0. \tag{10}$$

When the process by which equation (7) is obtained from the first of equations (6) is applied to equation (9), the result is

$$\lambda^{i}{}_{,j} = a\lambda^{i}\lambda_j + b\lambda^{i}\mu_j + c\mu^{i}\lambda_j. \tag{11}$$

When the first of equations (2) is differentiated covariantly with respect to x^j and in the resulting equation

$$\lambda_{1;}{}^{i}\lambda_{1;i,j} + \lambda_{1;i}\lambda_{1;}{}^{i}{}_{,j} = 0$$

$\lambda_{1;i,j}$ and $\lambda_{1;}{}^{i}{}_{,j}$ are replaced by their expressions from (6) and (7), the equation is satisfied identically in accordance with the first of equations (5). The result is the same when the second and third of equations (2) are differentiated covariantly with respect to x^j.

When the first of equations (3) is differentiated covariantly with respect to x^j and in the resulting equation $\lambda_{1;}{}^{i}{}_{,j}$ and $\lambda_{2;i,j}$ are replaced by these expressions from (7) and (6), the equation is satisfied identically in accordance with equations (5). The result is the same when the second and third of equations (3) are differentiated covariantly with respect to x^j.

When the first of equations (5) is differentiated covariantly with respect to x^j and when in the resulting equation

$$\lambda_i\lambda_{i;}{}^{i}{}_{,j} + \lambda_{1;}{}^{i}\lambda_{i,j} = 0$$

$\lambda_{1;}{}^{i}{}_{,j}$ and $\lambda_{i,j}$ are replaced by their expressions from (7) and (9), in accordance with equations (5) the result to within the factor $c\lambda_j$ is

$$\lambda_{1;}{}^{i}\mu_i = 0. \tag{12}$$

When the same process is applied to the second and third of equations (5), the result is

$$\lambda_{2;}{}^{i}\mu_{i} = 0, \quad \lambda_{3;}{}^{i}\mu_{i} = 0. \tag{13}$$

4. When the equation

$$\lambda_{1;\,i,j} = \lambda_{i}\lambda_{1;\,j}$$

from (6) is differentiated covariantly with respect to x^k and when in the resulting equation

$$\lambda_{1;\,i,jk} = \lambda_{i,k}\lambda_{1;\,j} + \lambda_{i}\lambda_{1;\,j,k}$$

$\lambda_{i,k}$ and $\lambda_{l;\,j,k}$ are replaced by their expressions of the form (9) and (6), the result is

$$\lambda_{1;\,i,jk} = \lambda_{1;\,j}(a\lambda_{i}\lambda_{k} + b\lambda_{i}\mu_{k} + c\mu_{i}\lambda_{k}) + \lambda_{i}\lambda_{j}\lambda_{;\,k}.$$

When j and k are interchanged, the resulting equation is

$$\lambda_{1;\,i,kj} = \lambda_{1;\,j}\lambda_{i}\lambda_{k} + \lambda_{1;\,k}(a\lambda_{i}\lambda_{j} + b\lambda_{i}\mu_{j} + c\mu_{i}\lambda_{j}).$$

When this equation is subtracted from the preceding one, the result is

$$\lambda_{1;\,i,jk} - \lambda_{1;\,i,kj} = \lambda_{1;\,j}[(a-1)\lambda_{i}\lambda_{k} + b\lambda_{i}\mu_{k} + c\mu_{i}\lambda_{k}] - \lambda_{1;\,k}[(a-1)\lambda_{i}\lambda_{j} + b\lambda_{i}\mu_{j} + c\mu_{i}\lambda_{j}]. \tag{14}$$

When in the equation[6]

$$\lambda_{1;\,h}R^{h}{}_{ijk} = \lambda_{1;\,i,jk} - \lambda_{1;\,i,kj}$$

the term $\lambda_{1;\,h}\lambda_{1;}{}^{h}$ which by (2) is equal to 1 is introduced as a multiplier of the right-hand member of this equation, the resulting equation is satisfied by

$$R^{h}{}_{ijk} = \lambda_{1;}{}^{h}(\lambda_{1;\,i,jk} - \lambda_{1;\,i,kj}),$$

which by means of (14) becomes

$$R^{h}{}_{ijk} = \lambda_{1;}{}^{h}\lambda_{1;\,j}[(a-1)\lambda_{i}\lambda_{k} + b\lambda_{i}\mu_{k} + c\mu_{i}\lambda_{k}] - \lambda_{1;}{}^{h}\lambda_{1;\,k}[(a-1)\lambda_{i}\lambda_{j} + b\lambda_{i}\mu_{j} + c\mu_{i}\lambda_{j}].$$

When this equation is contracted for h and k, in accordance with equations (5), the result is[7]

$$R_{ij} = \lambda_{1;\,j}b\lambda_{i}\lambda_{1;}{}^{h}\mu_{h} - [(a-1)\lambda_{i}\lambda_{j} + b\lambda_{i}\mu_{j} + c\mu_{i}\lambda_{j}].$$

The first term on the right is equal to zero in accordance with (12), and the resulting equation is

$$R_{ij} = (1-a)\lambda_{i}\lambda_{j} - b\lambda_{i}\mu_{j} - c\lambda_{j}\mu_{i}.$$

Since R_{ij} is symmetric in i and j,[8] c must be equal to b so that

$$R_{ij} = (1-a)\lambda_{i}\lambda_{j} - b(\lambda_{i}\mu_{j} + \lambda_{j}\mu_{i}). \tag{15}$$

When this equation is multiplied by g^{ij} and contracted for i and j, the resulting equation is[9]

$$R = g^{ij}R_{ij} = (1-a)\lambda_{1}\lambda^{i} - b(\lambda^{j}\mu_{j} + \lambda^{i}\mu_{i}).$$

In accordance with equations (4) and (12) the result is

$$R = 0. \tag{16}$$

5. For $c = b$ the right-hand member of equation (9) is symmetric in i and j, from

which it follows that

$$\lambda_{i,j} = \lambda_{j,i}. \tag{17}$$

In accordance with the equation[4]

$$\lambda_{i;j} = \frac{\partial \lambda_i}{\partial x^j} - \lambda_h \left\{ {h \atop ij} \right\} \tag{18}$$

and the fact that $\left\{ {h \atop ij} \right\}$ is symmetric in i and j, equation (17) reduces to

$$\frac{\partial \lambda_i}{\partial x^j} = \frac{\partial \lambda_j}{\partial x^i}.$$

This equation is satisfied by

$$\lambda_i = \frac{\partial \theta}{\partial x^i}, \tag{19}$$

where by equations (9) and (18), the function θ is a solution of the equations

$$\frac{\partial^2 \theta}{\partial x^i \partial x^j} = \left\{ {h \atop ij} \right\} \frac{\partial \theta}{\partial x^h} + a \frac{\partial \theta}{\partial x^i} \frac{\partial \theta}{\partial x^j} + b \left(\mu_j \frac{\partial \theta}{\partial x^i} + \mu_i \frac{\partial \theta}{\partial x^j} \right)$$

and by (4) satisfying the equation

$$g^{ij} \frac{\partial \theta}{\partial x^i} \frac{\partial \theta}{\partial x^j} = 0.$$

The orthogonal system of vectors λ_i defined by (19) and $\lambda_{1;i}$, $\lambda_{2;i}$ and $\lambda_{3;i}$ satisfying equations (2), (3), and (5) constitutes the solution of the problem of the present paper.

[1] These PROCEEDINGS, **46**, 1093 (1960).

[2] Einstein, A., *The Meaning of Relativity*, 3rd ed. (Princeton University Press, 1950), p. 92.

[3] *R. G.*, p. 38. A reference of this type is to the author's *Riemannian Geometry* (Princeton University Press, 1949).

[4] *R. G.*, equation (11.3), p. 27.

[5] *R. G.*, equation (11.8), p. 28.

[6] *R. G.*, equation (11.14), p. 30.

[7] *R. G.*, equation (8.14), p. 21.

[8] *R. G.*, pp. 21, 22.

[9] *R. G.*, equation (26.5), p. 83.

GENERALIZED SPACES OF GENERAL RELATIVITY, II

By Luther Pfahler Eisenhart

PRINCETON UNIVERSITY

Communicated October 26, 1960

1. This paper is a revision of the paper with the same title which was published a year ago.[1]

The paper deals with a space of four dimensions V_4 of coordinates x^i as i takes the values 1 to 4. The geometry of the space is based upon the asymmetric functions g_{ij} and the asymmetric coefficients of connection Γ^h_{ij} and upon the Einstein equation[2]

$$g_{ij,k} = g_{ih}\,\Gamma^h_{kj} + g_{hj}\,\Gamma^h_{ik}. \tag{1}$$

Here, $g_{ij,k}$ denotes the derivative of g_{ij} with respect to x^k and each of the terms on the right stands for the sum of terms as h takes the values 1 to 4.

Throughout this paper, a quantity followed by a comma and index denotes the derivative of the quantity with respect to x with this index. Also, when there is a repeated upper and lower index, the one term stands for the sum of terms as that index takes the values 1 to 4, as for example h in equation (1).

Write equation (1) in the form

$$g_{\underline{ij},k} + g_{\underset{\sim}{ij},k} = (g_{\underline{ih}} + g_{\underset{\sim}{ih}})(\Gamma^h_{\underline{jk}} - \Gamma^h_{\underset{\sim}{jk}}) + (g_{\underline{jh}} - g_{\underset{\sim}{jh}})(\Gamma^h_{\underline{ik}} + \Gamma^k_{\underset{\sim}{ik}}),$$

where $g_{\underline{ij}}$ and $g_{\underset{\sim}{ij}}$ are the symmetric and skew-symmetric parts of g_{ij}, and $\Gamma^h_{\underline{jk}}$ and $\Gamma^h_{\underset{\sim}{jk}}$ are the symmetric and skew-symmetric parts of Γ^h_{jk}. This type of notation is used throughout this paper. When i and j are interchanged in the above equation, the resulting equation is

$$g_{\underline{ij},k} - g_{\underset{\sim}{ij},k} = (g_{\underline{ih}} - g_{\underset{\sim}{ih}})(\Gamma^h_{\underline{jk}} + \Gamma^h_{\underset{\sim}{jk}}) + (g_{\underline{jh}} + g_{\underset{\sim}{jh}})(\Gamma^h_{\underline{ik}} - \Gamma^h_{\underset{\sim}{ik}}).$$

One-half the sum and one-half the difference of these two equations are the equations

$$g_{\underline{ij},k} = g_{\underline{ih}}\,\Gamma^h_{\underline{jk}} + g_{\underline{jh}}\,\Gamma^h_{\underline{ik}} - g_{\underline{ih}}\,\Gamma^h_{\underset{\sim}{jk}} - g_{\underline{jh}}\,\Gamma^h_{\underset{\sim}{ik}}, \tag{2}$$

$$g_{\underset{\sim}{ij},k} = g_{\underline{ih}}\,\Gamma^h_{\underset{\sim}{jk}} - g_{\underline{jh}}\,\Gamma^h_{\underline{ik}} - g_{\underline{ih}}\,\Gamma^h_{\underline{jk}} + g_{\underline{jh}}\,\Gamma^h_{\underline{ik}}. \tag{3}$$

We impose upon the skew-symmetric Γ's conditions of the type

$$g_{\underline{ih}}\,\Gamma^h_{\underset{\sim}{jk}} + g_{\underline{jh}}\,\Gamma^h_{\underset{\sim}{ik}} = 0, \tag{4}$$

and equation (2) becomes

$$g_{\underline{ij},k} = g_{\underline{ih}}\,\Gamma^h_{\underline{jk}} + g_{\underline{jh}}\,\Gamma^h_{\underline{ik}}. \tag{5}$$

From equations of this form, we have

$$g_{\underline{ik},j} + g_{\underline{jk},i} - g_{\underline{ij},k} = 2g_{\underline{hk}}\,\Gamma^h_{\underline{ij}}.$$

From the form of this equation, it follows[3] that $\Gamma^h_{\underline{ij}}$ is the Christoffel symbol $\left\{ {h \atop ij} \right\}$ in terms of the symmetric g's. Accordingly, we write equation (3) in the form

$$g_{\underset{\sim}{ij},k} = g_{\underset{\sim}{ih}} \left\{ {h \atop jk} \right\} - g_{\underset{\sim}{jh}} \left\{ {h \atop ik} \right\} - g_{\underline{ih}} \Gamma^h_{\underset{\sim}{jk}} + g_{\underline{jh}} \Gamma^h_{\underset{\sim}{ik}} \tag{6}$$

and equation (5) in the form

$$g_{ij,k} = g_{\underline{ih}} \left\{ {h \atop jk} \right\} + g_{\underline{jh}} \left\{ {h \atop ik} \right\}. \tag{7}$$

When equation (6) is differentiated with respect to x^l and the derivatives of the skew-symmetric g's and the symmetric g's are replaced by means of the equations of the types (6) and (7), the result is

$$\begin{aligned} g_{\underset{\sim}{ij},kl} = \quad & g_{\underline{im}} \left(\left\{ {m \atop jk} \right\},l + \left\{ {h \atop jk} \right\} \left\{ {m \atop hl} \right\} \right) - g_{\underline{jm}} \left(\left\{ {m \atop ik} \right\},l + \left\{ {h \atop ik} \right\} \left\{ {m \atop hl} \right\} \right) \\ & - g_{\underline{im}} \left(\Gamma^m_{\underset{\sim}{jk},l} + \left\{ {h \atop jk} \right\} \Gamma^m_{\underset{\sim}{hl}} + \left\{ {m \atop hl} \right\} \Gamma^h_{\underset{\sim}{jk}} \right) + g_{\underline{jm}} \left(\Gamma^m_{\underset{\sim}{ik},l} + \left\{ {h \atop ik} \right\} \Gamma^m_{\underset{\sim}{hl}} + \left\{ {m \atop hl} \right\} \Gamma^h_{\underset{\sim}{ij}} \right) \\ & + g_{hm} \left(\left\{ {h \atop ik} \right\} \left\{ {m \atop jl} \right\} - \left\{ {h \atop jk} \right\} \left\{ {m \atop il} \right\} \right) - g_{\underline{hm}} \left(\left\{ {h \atop jk} \right\} \Gamma^m_{il} - \left\{ {h \atop ik} \right\} \Gamma^m_{jl} + \left\{ {m \atop jl} \right\} \Gamma^h_{ik} - \left\{ {m \atop il} \right\} \Gamma^h_{\underset{\sim}{ij}} \right). \end{aligned} \tag{8}$$

When the equation obtained from equation (8) by interchanging k and l is subtracted from equation (8), the result is

$$\begin{aligned} 0 = \quad & g_{\underline{im}} \left(\left\{ {m \atop jk} \right\},l - \left\{ {m \atop jl} \right\},k + \left\{ {h \atop jk} \right\} \left\{ {m \atop hl} \right\} - \left\{ {h \atop jl} \right\} \left\{ {m \atop hk} \right\} \right) \\ & - g_{\underline{jm}} \left(\left\{ {m \atop ik} \right\},l - \left\{ {m \atop il} \right\},k + \left\{ {h \atop ik} \right\} \left\{ {m \atop hl} \right\} - \left\{ {h \atop il} \right\} \left\{ {m \atop hk} \right\} \right) \\ & - g_{\underline{im}} \left(\Gamma^m_{\underset{\sim}{jk},l} - \Gamma^m_{\underset{\sim}{jl},k} + \left\{ {h \atop jk} \right\} \Gamma^m_{\underset{\sim}{hl}} - \left\{ {h \atop jl} \right\} \Gamma^m_{\underset{\sim}{hk}} + \left\{ {m \atop hl} \right\} \Gamma^h_{\underset{\sim}{jk}} - \left\{ {m \atop hk} \right\} \Gamma^h_{\underset{\sim}{jl}} \right) \\ & + g_{\underline{jm}} \left(\Gamma^m_{\underset{\sim}{ik},l} - \Gamma^m_{\underset{\sim}{il},k} + \left\{ {h \atop ik} \right\} \Gamma^m_{\underset{\sim}{hl}} - \left\{ {h \atop il} \right\} \Gamma^m_{\underset{\sim}{hk}} + \left\{ {m \atop hl} \right\} \Gamma^h_{\underset{\sim}{ik}} - \left\{ {m \atop hk} \right\} \Gamma^h_{\underset{\sim}{il}} \right). \end{aligned} \tag{9}$$

The covariant derivative of $\Gamma^m_{\underset{\sim}{jk}}$ with respect to x^l denoted by $\Gamma^m_{\underset{\sim}{jk};l}$ is

$$\Gamma^m_{\underset{\sim}{jk};l} = \Gamma^m_{\underset{\sim}{jk},l} + \left\{ {m \atop hl} \right\} \Gamma^h_{\underset{\sim}{jk}} - \left\{ {h \atop jl} \right\} \Gamma^m_{\underset{\sim}{hk}} - \left\{ {h \atop kl} \right\} \Gamma^m_{\underset{\sim}{jh}}.$$

When the equation obtained by interchanging k and l in this equation is subtracted from this equation, the result is the equation

$$\Gamma^m_{\underset{\sim}{jk},l} - \Gamma^m_{\underset{\sim}{jl},k} = \Gamma^m_{\underset{\sim}{jk},l} - \Gamma^m_{\underset{\sim}{jl},k} + \left\{ {h \atop jk} \right\} \Gamma^m_{\underset{\sim}{hl}} - \left\{ {h \atop jl} \right\} \Gamma^m_{\underset{\sim}{hk}} + \left\{ {m \atop kl} \right\} \Gamma^h_{\underset{\sim}{jk}} - \left\{ {m \atop jk} \right\} \Gamma^h_{\underset{\sim}{jl}}.$$

Since the right hand member of the equation is the same as the quantity in parentheses in the third line of equation (9) and the quantity in parentheses in the first line of equation (9) is equal to the Riemannian tensor R^m_{jlk} (see ref. 5), equation (9) is equal to the equation

$$g_{\underline{im}} R^m_{\underset{\sim}{jlk}} - g_{\underline{jm}} R^m_{\underset{\sim}{ilk}} - g_{\underline{im}} \left(\Gamma^m_{\underset{\sim}{jk};l} - \Gamma^m_{\underset{\sim}{jl};k} \right) + g_{\underline{jm}} \left(\Gamma^m_{\underset{\sim}{ik};l} - \Gamma^m_{\underset{\sim}{il};k} \right). \tag{10}$$

Since the covariant derivative $g_{\underline{im}}$ is equal to zero,[6] we have

$$g_{\underline{im}} \Gamma^m_{\underset{\sim}{jk};l} = \left(g_{\underline{im}} \Gamma^m_{\underset{\sim}{jk}} \right)_{;l} = \Gamma_{\underset{\sim}{ijk};l}.$$

By means of equation of this type, equation (10) becomes

$$g_{\underline{im}} R^m_{\underset{\sim}{jlk}} + g_{\underline{mj}} R^m_{\underset{\sim}{ilk}} - \Gamma_{\underset{\sim}{ijk};l} + \Gamma_{\underset{\sim}{ijl};k} + \Gamma_{\underset{\sim}{jlk};l} - \Gamma_{\underset{\sim}{jil};k} = 0. \tag{11}$$

When equation (4), which led to equation (5), from which it follows that $\Gamma^h_{\underline{ij}}$ is the Christoffel symbol $\left\{ {h \atop ij} \right\}$ in terms of the symmetric g's, is written in the form

$$g_{ih}\, g^{ih}\, \Gamma_{ijk} + g_{jh}\, g^{ih}\, \Gamma_{iik} = 0,$$

it is seen that the tensor Γ_{ijk} is skew-symmetric in each pair of indices. Accordingly, equation (11) may be written in the form

$$g_{im}\, R^m_{jlk} + g_{mj}\, R^m_{ilk} - 2\Gamma_{ijk;l} + 2\Gamma_{ijl;k} = 0. \tag{12}$$

The skew-symmetric g^{ij} is by definition such that

$$g^{ij}\, g_{ik} = g^{ji}\, g_{ki} = \delta^j_k, \tag{13}$$

where δ^k_j is the Kronecker delta, where

$$\delta^i_i = 1,\ \delta^i_j = 0, \tag{14}$$

in the first of which i is not summed.

When equation (12) is multiplied by g^{ij} and summed for i and j, the resulting equation is[7]

$$g^{ij}\, g_{im}\, R^m_{jlk} + g^{ij}\, g_{mj}\, R^m_{ilk} = 2g^{ij}\, (\Gamma_{ijk;l} - \Gamma_{ijl;k}),$$

which by means of (14) becomes

$$R^j_{jlk} + R^i_{ilk} = 2g^{ij}\, (\Gamma_{ijk;l} - \Gamma_{ijl;k}).$$

Since $R^i_{ilk} = 0$,[8] we have to within the factor 2

$$g^{ij}\, \Gamma_{ijk;l} - g^{ij}\, \Gamma_{ijl;k} = 0 \tag{15}$$

The geometry of the space is based upon the Einstein equation.[2] By means of the condition (4), which we impose on the skew-symmetric parts Γ^h_{ij} of the coefficient of connection, it follows that the symmetric parts Γ^h_{ij} become the Christoffel symbols of the symmetric g's. The skew-symmetric Γ's in the form Γ_{ijk} are skew-symmetric in each pair of indices, and their covariant derivatives are in the relation of equation (15). The derivation of these results is the purpose of the paper.

[1] These PROCEEDINGS, **45**, 1759–1762 (1959).

[2] Einstein, A., *The Meaning of Relativity* (Princeton University Press, 1954), equation (12), p. 138 and equation (29), p. 144.

[3] *R. G.*, equations (7.3) and (7.1), p. 17. A reference of this form is to the author's *Riemannian Geometry* (Princeton University Press, 1949).

[4] *R. G.*, equation (11.7), p. 28.

[5] *R. G.*, equation (8.3), p. 19.

[6] *R. G.*, equation (11.8), p. 28.

[7] *R. G.*, equation (8.5), p. 20.

[8] *R. G.*, equation (8.13), p. 21 for $1 = i$.

SPACES WHICH ADMIT FIELDS OF NORMAL NULL VECTORS

By Luther P. Eisenhart

PRINCETON UNIVERSITY

Communicated September 29, 1960

1. We denote by $\lambda_{h|}{}^{i}$ n in contravariant null vectors, as h takes the values 1 to n, that is

$$g_{ij}\lambda_{h|}{}^{i}\lambda_{h|}{}^{j} = 0, \tag{1}$$

where g_{ij} are the components of the metric tensor of the space.

Here and throughout this paper when in an expression there is the same upper and lower index, as i and j in (1), the term stands for the sum of terms as the index takes the values 1 to n.

We write equation (1) in the form

$$\lambda_{h|}{}^{i}\lambda_{h|i} = 0. \tag{2}$$

We introduce the equation

$$\lambda_{h|i,j} = \lambda_{h|i}\lambda_{h|j} + \lambda_{h|i}\mu_{h|j} + \lambda_{h|j}\mu_{h|i} \tag{3}$$

where $\mu_{h|i}$ are the covariant components of a vector to be specified later and $\lambda_{h|i,j}$ denotes the covariant derivative with respect to x^j of the vector $\lambda_{h|i}$. This notation is used throughout this paper.

Since the right-hand expression in equation (3) is symmetric in i and j, it follows that

$$\lambda_{h|i,j} = \lambda_{h|j,i},$$

from which since[1]

$$\lambda_{h|i,j} = \frac{\partial\lambda_{h|i}}{\partial x^j} - \lambda_{h|k}\left\{ {k \atop ij} \right\}$$

and $\left\{ {k \atop ij} \right\}$ is symmetric in i and j, we have

$$\frac{\partial\lambda_{h|i}}{\partial x^j} = \frac{\partial\lambda_{h|j}}{\partial x^i}.$$

This equation is satisfied by

$$\lambda_{h|i} = \frac{\partial\varphi_h}{\partial x^i}, \tag{4}$$

where φ_h is a function of the x's, that is, the $\lambda_{h|i}$ are the components of the normals to the hyperspaces

$$\varphi_h = \text{const.} \tag{5}$$

2. Since the covariant derivative of g^{ik} is equal to zero,[2] when equation (3) is multiplied by g^{ik} and summed for i, the result is

$$(g^{ik}\lambda_{h|i})_{,j} = \lambda_{h|}{}^{k}{}_{,j} = \lambda_{h|}{}^{k}\lambda_{h|j} + \lambda_{h|}{}^{k}\mu_{h|j} + \lambda_{h|j}\mu_{h|}{}^{k}.$$

$$g_{ih}\, g^{ih}\, \Gamma_{ijk} + g_{jh}\, g^{jh}\, \Gamma_{jik} = 0,$$

it is seen that the tensor Γ_{ijk} is skew-symmetric in each pair of indices. Accordingly, equation (11) may be written in the form

$$g_{im}\, R^m_{jlk} + g_{mj}\, R^m_{ilk} - 2\Gamma_{ijk;l} + 2\Gamma_{ijl;k} = 0. \tag{12}$$

The skew-symmetric g^{ij} is by definition such that

$$g^{ij}\, g_{ik} = g^{ji}\, g_{ki} = \delta^j_k, \tag{13}$$

where δ^k_j is the Kronecker delta, where

$$\delta^i_i = 1,\ \delta^i_j = 0, \tag{14}$$

in the first of which i is not summed.

When equation (12) is multiplied by g^{ij} and summed for i and j, the resulting equation is[7]

$$g^{ij}\, g_{im}\, R^m_{jlk} + g^{ij}\, g_{mj}\, R^m_{ilk} = 2g^{ij}\, (\Gamma_{ijk;l} - \Gamma_{ijl;k}),$$

which by means of (14) becomes

$$R^j_{jlk} + R^i_{ilk} = 2g^{ij}\, (\Gamma_{ijk;l} - \Gamma_{ijl;k}).$$

Since $R^i_{ilk} = 0$,[8] we have to within the factor 2

$$g^{ij}\, \Gamma_{ijk;l} - g^{ij}\, \Gamma_{ijl;k} = 0 \tag{15}$$

The geometry of the space is based upon the Einstein equation.[2] By means of the condition (4), which we impose on the skew-symmetric parts Γ^h_{ij} of the coefficient of connection, it follows that the symmetric parts Γ^h_{ij} become the Christoffel symbols of the symmetric g's. The skew-symmetric Γ's in the form Γ_{ijk} are skew-symmetric in each pair of indices, and their covariant derivatives are in the relation of equation (15). The derivation of these results is the purpose of the paper.

[1] These PROCEEDINGS, **45**, 1759–1762 (1959).

[2] Einstein, A., *The Meaning of Relativity* (Princeton University Press, 1954), equation (12), p. 138 and equation (29), p. 144.

[3] *R. G.*, equations (7.3) and (7.1), p. 17. A reference of this form is to the author's *Riemannian Geometry* (Princeton University Press, 1949).

[4] *R. G.*, equation (11.7), p. 28.

[5] *R. G.*, equation (8.3), p. 19.

[6] *R. G.*, equation (11.8), p. 28.

[7] *R. G.*, equation (8.5), p. 20.

[8] *R. G.*, equation (8.13), p. 21 for $1 = i$.

SPACES WHICH ADMIT FIELDS OF NORMAL NULL VECTORS

By Luther P. Eisenhart

PRINCETON UNIVERSITY

Communicated September 29, 1960

1. We denote by $\lambda_{h|}{}^{i}$ n in contravariant null vectors, as h takes the values 1 to n, that is

$$g_{ij}\lambda_{h|}{}^{i}\lambda_{h|}{}^{j} = 0, \tag{1}$$

where g_{ij} are the components of the metric tensor of the space.

Here and throughout this paper when in an expression there is the same upper and lower index, as i and j in (1), the term stands for the sum of terms as the index takes the values 1 to n.

We write equation (1) in the form

$$\lambda_{h|}{}^{i}\lambda_{h|i} = 0. \tag{2}$$

We introduce the equation

$$\lambda_{h|i,j} = \lambda_{h|i}\lambda_{h|j} + \lambda_{h|i}\mu_{h|j} + \lambda_{h|j}\mu_{h|i} \tag{3}$$

where $\mu_{h|i}$ are the covariant components of a vector to be specified later and $\lambda_{h|i,j}$ denotes the covariant derivative with respect to x^j of the vector $\lambda_{h|i}$. This notation is used throughout this paper.

Since the right-hand expression in equation (3) is symmetric in i and j, it follows that

$$\lambda_{h|i,j} = \lambda_{h|j,i},$$

from which since[1]

$$\lambda_{h|i,j} = \frac{\partial\lambda_{h|i}}{\partial x^j} - \lambda_{h|k}\left\{ {k \atop ij} \right\}$$

and $\left\{ {k \atop ij} \right\}$ is symmetric in i and j, we have

$$\frac{\partial\lambda_{h|i}}{\partial x^j} = \frac{\partial\lambda_{h|j}}{\partial x^i}.$$

This equation is satisfied by

$$\lambda_{h|i} = \frac{\partial\varphi_h}{\partial x^i}, \tag{4}$$

where φ_h is a function of the x's, that is, the $\lambda_{h|i}$ are the components of the normals to the hyperspaces

$$\varphi_h = \text{const.} \tag{5}$$

2. Since the covariant derivative of g^{ik} is equal to zero,[2] when equation (3) is multiplied by g^{ik} and summed for i, the result is

$$(g^{ik}\lambda_{h|i})_{,j} = \lambda_{h|}{}^{k}{}_{,j} = \lambda_{h|}{}^{k}\lambda_{h|j} + \lambda_{h|}{}^{k}\mu_{h|j} + \lambda_{h|j}\mu_{h|}{}^{k}.$$

When k is replaced by i, the result is

$$\lambda_{h|}{}^{t}{}_{,j} = \lambda_{h|}{}^{t}\lambda_{h|j} + \lambda_{h|}{}^{t}\mu_{h|j} + \lambda_{h|j}\mu_{h|}{}^{t}. \tag{6}$$

When equation (3) is multiplied by $\lambda_{h|}{}^{t}$ and summed for i, by means of equation (2) the result is

$$\lambda_{h|}{}^{t}\lambda_{h|i,j} = \lambda_{h|j}\lambda_{h|}{}^{t}\mu_{h|i}.$$

When equation (6) is multiplied by $\lambda_{h|i}$ and summed for i, the result is

$$\lambda_{h|i}\lambda_{h|}{}^{i}{}_{,j} = \lambda_{h|j}\lambda_{h|i}\mu_{h|}{}^{i}.$$

When these expressions for $\lambda_{h|}{}^{t}\lambda_{h|i,j}$ and $\lambda_{h|i}\lambda_{h|}{}^{i}{}_{,j}$ are substituted in the equation

$$\lambda_{h|}{}^{i}{}_{,j}\lambda_{h|i} + \lambda_{h|i,j}\lambda_{h|}{}^{i} = 0 \tag{7}$$

which is obtained by differentiating covariantly with respect to x^j equation (2), to within a factor 2 the result is

$$\lambda_{h|i}\mu_{h|}{}^{i} = \lambda_{h|}{}^{i}\mu_{h|i} = 0. \tag{8}$$

Hence the vectors $\lambda_{h|i}$ and $\mu_{h|i}$ are orthogonal to one another.[3]

When the second of equations (8) is differentiated covariantly with respect to x^j and in the resulting equation

$$\lambda_{h|}{}^{i}\mu_{h|i,j} + \mu_{h|i}\lambda_{h|}{}^{i}{}_{,j} = 0$$

the quantity $\lambda_{h|}{}^{i}{}_{,j}$ is replaced by its expression from equation (6), by means of equation (8) the result is

$$\lambda_{h|}{}^{i}\mu_{h|i,j} + \mu_{h|i}\mu_{h|}{}^{i}\lambda_{h|j} = 0. \tag{9}$$

3. When equation (3) is differentiated covariantly with respect to x^k and in the resulting equation

$$\lambda_{h|i,jk} = \lambda_{h|i,k}\lambda_{h|j} + \lambda_{h|i}\lambda_{h|j,k} + \lambda_{h|i,k}\mu_{h|j} + \lambda_{h|i}\mu_{h|j,k} + \lambda_{h|j,k}\mu_{h|i} + \lambda_{h|j}\mu_{h|i,k}$$

the quantities $\lambda_{h|i,k}$ are replaced by their expressions of the type (3), the result is

$$\lambda_{h|i,jk} = \lambda_{h|j}(\lambda_{h|i}\lambda_{h|k} + \lambda_{h|i}\mu_{h|k} + \lambda_{h|k}\mu_{h|i}) + \lambda_{h|i}\lambda_{h|j,k} + \lambda_{h|i}\mu_{h|j,k} + \mu_{h|j}(\lambda_{h|i}\lambda_{h|k} + \lambda_{h|i}\mu_{h|k} + \lambda_{h|k}\mu_{h|i}) + \lambda_{h|j}\mu_{h|i,k}.$$

When j and k are interchanged in this equation and the resulting equation is subtracted from the above equation, since $\lambda_{h|j,k} = \lambda_{h|k,j}$ the result is

$$\lambda_{h|i,jk} - \lambda_{h|i,kj} = \lambda_{h|j}(\mu_{h|i,k} - \mu_{h|i}\mu_{h|k}) - \lambda_{h|k}(\mu_{h|i,j} - \mu_{h|i}\mu_{h|j}) + \lambda_{h|i}(\mu_{h|j,k} - \mu_{h|k,j}). \tag{10}$$

4. We put

$$\mu_{h|i,j} = \mu_{h|i}\mu_{h|j} + a\lambda_{h|i}\mu_{h|j} + b\lambda_{h|j}\mu_{h|i} + c\mu_{h|i}\mu_{h|j}, \tag{11}$$

where a, b, and c are constants. For expressions of this type equation (10) becomes

$$\lambda_{h|i,jk} - \lambda_{h|i,kj} = [(2a - b)\lambda_{h|i} + c\mu_{h|i}]\,(\lambda_{h|j}\mu_{h|k} - \lambda_{h|k}\mu_{h|j}). \tag{12}$$

By means of the equation[4]

$$\lambda_{h|i,jk} - \lambda_{h|i,kj} = \lambda_{h|l}R^{l}{}_{ijk}$$

equation (12) becomes

$$\lambda_{h|l}R^{l}_{ijk} = [(2a - b)\lambda_{h|i} + c\mu_{h|i}](\lambda_{h|j}\mu_{h|k} - \lambda_{h|k}\mu_{h|j}).$$

We sum this equation for h and write the result in the form

$$\sum_h \lambda_{h|l}R^{l}_{ijk} = (2a - b)\sum_h \lambda_{h|i}\lambda_{h|j}\mu_{h|k} - (2a - b)\sum_h \lambda_{h|i}\lambda_{h|k}\mu_{h|j} + c\sum_h \mu_{h|i}\lambda_{h|j}\mu_{h|k} - c\sum_h \mu_{h|i}\mu_{h|j}\lambda_{h|k}. \quad (13)$$

We introduce a vector γ^l by the equation

$$\gamma^l \sum_h \lambda_{h|l} = 1, \quad (14)$$

and insert $\gamma^l \sum_h \lambda_{h|l}$ as a multiplier of each of the four terms of the right-hand member of equation (13). When the factor $\sum_h \lambda_{h|l}$ is dropped from each term of the resulting equation, the following equation results

$$R^{l}_{ijk} = (2a - b)\gamma^l \sum_h \lambda_{h|i}\lambda_{h|j}\mu_{h|k} - (2a - b)\gamma^l \sum_h \lambda_{h|i}\lambda_{h|k}\mu_{h|j} + c\gamma^l \sum_h \mu_{h|i}\lambda_{h|j}\mu_{h|k} - c\gamma^l \sum_h \mu_{h|i}\mu_{h|j}\lambda_{h|k}.$$

When this equation is contracted for l and k, the result is[5]

$$R_{ij} = (2a - b)\sum_h \lambda_{h|i}\lambda_{h|j}\gamma^l\mu_{h|l} - (2a - b)\sum_h \lambda_{h|i}\mu_{h|j}\gamma^l\lambda_{h|l} + c\sum_h \mu_{h|i}\lambda_{h|j}\gamma^l\mu_{h|l} - c\sum_h \mu_{h|i}\mu_{h|j}\gamma^l\lambda_{h|l}. \quad (15)$$

When i and j are interchanged, the result is

$$R_{ji} = (2a - b)\sum_h \lambda_{h|i}\lambda_{h|j}\gamma^l\mu_{h|l} + c\sum_h \lambda_{h|i}\mu_{h|j}\gamma^l\lambda_{h|l} - (2a - b)\sum_h \mu_{h|i}\lambda_{h|j}\gamma^l\mu_{h|l} - c\sum_h \mu_{h|i}\mu_{h|j}\gamma^l\lambda_{h|l}. \quad (16)$$

We take

$$\gamma^l\mu_{h|l} = \gamma^l\lambda_{h|l}, \qquad c = 2a - b \quad (17)$$

by means of which and the fact that R_{ij} is symmetric in i and j[5], one half the sum of equations (15) and (16) is

$$R_{ij} = (2a - b)(\sum_h \lambda_{h|i}\lambda_{h|j}\gamma^l\lambda_{h|l} - \sum_h \mu_{h|i}\mu_{h|j}\gamma^l\mu_{h|l}). \quad (18)$$

5. By means of the second of equation (17) equation (11) becomes

$$\mu_{h|i,j} = (1 + 2a - b)\mu_{h|i}\mu_{h|j} + a\lambda_{h|i}\mu_{h|j} + b\lambda_{h|j}\mu_{h|i}. \quad (19)$$

When this equation is multiplied by $\lambda_{h|}{}^{i}$ and summed for i, in accordance with equations (2) and (8) the result is

$$\lambda_{h|}{}^{i}\mu_{h|i,j} = 0. \quad (20)$$

By means of this equation the equation (9) becomes to within the factor $\lambda_{h|j}$

$$\mu_{h|i}\mu_{h|}{}^{i} = 0, \tag{21}$$

that is $\mu_{h|i}$ is a null-vector.

In accordance with this result and equation (2) when equation (18) is multiplied by g^{ij} and summed for i and j, the result is[6]

$$R = g^{ij}R_{ij} = 0. \tag{22}$$

6. When the process by which equation (6) is obtained from equation (3) is applied to equation (19) the result is

$$\mu_{h|}{}^{i}{}_{,j} = (1 + 2a - b)\mu_{h|}{}^{i}\mu_{h|j} + a\lambda_{h|}{}^{i}\mu_{h|j} + b\lambda_{h|j}\mu_{h|}{}^{i}. \tag{23}$$

When equation (19) is multiplied by $\mu_{h|}{}^{i}$ and summed for i, in accordance with equation (8) and (21) the result is

$$\mu_{h|}{}^{i}\mu_{h|i,j} = 0.$$

In like manner from equation (23) we obtain

$$\mu_{h|i}\mu_{h|}{}^{i}{}_{,j} = 0.$$

In accordance with these equations, the equation

$$\mu_{h|}{}^{i}\mu_{h|i,j} + \mu_{h|i}\mu_{h|}{}^{i}{}_{,j} = 0$$

obtained by differentiating equation (21) covariantly with respect to x^j is satisfied identically.

[1] *R.G.*, equation (11.3), p. 27. A reference of this form is to the author's *Riemannian Geometry*, (Princeton University Press, 1949).
[2] *R.G.*, equation (11.8), p. 28.
[3] *R.G.*, equation (13.12), p. 40.
[4] *R.G.*, equation (11.14), p. 30.
[5] *R.G.*, equation (8.14), p. 21.
[6] *R.G.*, equation (26.5), p. 83.

A METHOD FOR DEFINING PRINCIPAL MODES OF NONLINEAR SYSTEMS UTILIZING INFINITE DETERMINANTS

By Demetrios G. Magiros

REPUBLIC AVIATION CORPORATION, FARMINGDALE, N. Y.

Communicated by L. Brillouin, October 3, 1960

1. The concept of "principal modes" plays the predominant role in the analysis of oscillatory systems, no matter what field the systems occur in. For the calculations of principal modes of systems, either linear or nonlinear, two appropriate definitions may be used, namely, the "proportionality definition" of principal modes and their definition as solutions of special "initial value problems."

It is the purpose of this note to provide a procedure for the calculation of principal

modes of nonlinear systems utilizing "infinite determinants." For a "two-mass-three-spring" free system with nonlinear coupling, R. Rosenberg[1] carried out the calculation of principal modes by using the "potential function" of the corresponding conservative system. We restrict ourselves, without loss of generality, to the above "dual-mode" nonlinear system for reasons of comparison with the results obtained by using other methods. The motion of this system is governed by two second-order ordinary differential equations one of which is nonlinear. When the solutions of these equations are assumed to have the form of complex exponential series, the calculation of the frequency and the coefficients leads to recursion formulae, which give rise to "infinite determinants."

2. In this note, the following "proportionality definition" of "principal modes" of an oscillatory nonlinear system is used. By using the terminology of mechanical systems, the "principal modes" are defined as those oscillations for which the amplitudes of the fundamental and the corresponding harmonics of the displacements of any two of the oscillating masses have, separately, a constant ratio at all times. For such motions, the masses all oscillate about their equilibrium positions with the same frequency, the "principal frequency" of the system. If x_i, $i = 1, 2, \ldots s$ are the displacements of the masses m_i, $i = 1, 2, \ldots s$ from their equilibrium positions and x_{in}, $x_{i'n}$ the corresponding amplitudes of the nth harmonic of the displacements x_i, $x_{i'}$ of the two masses m_i, $m_{i'}$, and if there exist constants c_n such that the conditions,

$$x_{in}/x_{i'n}(t) \equiv c_n, \; i \neq i', \; i, i' = 1, 2, \ldots s \tag{1}$$

are satisfied, then these motions are, by definition, the "principal modes" of the system.

3. If m_1 and m_2 are the oscillating masses, K_1 and K_3 the spring constants of the anchor springs, $\bar{K}_2 = K_2 + \mu x^2$ that of the coupling, μ a constant which characterizes the nonlinearity, and x and y the displacements of m_1 and m_2 from their equilibrium positions, the equations of motion of this system are

$$\begin{aligned} \ddot{x} + \omega_1^2 x + \lambda_1 x^3 - \lambda_2 y &= 0 \\ \ddot{y} + \omega_2^2 y - \lambda_3 x &= 0 \end{aligned} \tag{2}$$

where

$$\omega_1^2 = \frac{K_1 + K_2}{m_1}, \; \omega_2^2 = \frac{K_2 + K_3}{m_2}, \; \lambda_1 = \frac{\mu}{m_1}, \; \lambda_2 = \frac{K_2}{m_1}, \; \lambda_3 = \frac{K_2}{m_2}. \tag{2a}$$

Assume solution of (2) to be represented by the exponential series,

$$x(t) = \sum_{n=-\infty}^{\infty} \alpha_n e^{in\omega t}, \quad y(t) = \sum_{n=-\infty}^{\infty} A_n e^{in\omega t}. \tag{3}$$

For the reality of the solution, one takes $\bar{\alpha}_n = \alpha_{-n}$, $\bar{A}_n = A_{-n}$, where $\bar{\alpha}_n$ and $\bar{A}_n$ are conjugates of α_n and A_n. Also assume that

$$\sum_{n=-\infty}^{\infty} |\alpha_n| < \infty, \; \sum_{n=-\infty}^{\infty} |A_n| < \infty, \; \sum_{n=-\infty}^{\infty} n^2 |\alpha_n| < \infty, \; \sum_{n=-\infty}^{\infty} n^2 |A_n| < \infty.$$

The first two of these inequalities guarantee the convergence of the series (3),

while the second ones the existence of the second derivatives $\ddot{x}$, $\ddot{y}$. Inserting (3) into (2), if one puts x^3 into the form,

$$x^3 = \sum_n \sum_{\rho_1} \sum_{\rho_2} \alpha_{\rho_1}\alpha_{\rho_2}\alpha_{n-\rho_1-\rho_2} e^{in\omega t},$$

one can get the system,

$$\left(\omega_1^2 - n^2\omega^2 - \frac{\lambda_2\lambda_3}{\omega_2^2 - n^2\omega^2}\right)\alpha_n + \lambda_1 \sum_{\rho_1} \sum_{\rho_2} \alpha_{\rho_1}\alpha_{\rho_2}\alpha_{n-\rho_1-\rho_2} = 0 \qquad (4i)$$

$$A_n/\alpha_n = \frac{\lambda_3}{\omega_2^2 - n^2\omega^2} \qquad (4ii)$$

where n, ρ_1, ρ_2 are integers. This system consists of infinitely many nonlinear ($\lambda_1 \neq 0$) homogeneous equations for the infinitely many unknown coefficients α_n and A_n.

If α_1 is the dominant coefficient of the first of the series (3), the approximate value of the double series in (4i) is

$$c_1\alpha_1^2\alpha_{n-2} + c_2|\alpha_1|^2\alpha_n + c_3\bar{\alpha}_1^2\alpha_{n+2}$$

with an error $o(\alpha_1^2)$. c_1, c_2, c_3 are appropriately calculated integers. Then one can get from (4i) the following recursion formula:

$$p_n\alpha_{n-2} + \alpha_n + q_n\alpha_{n+2} = 0 \qquad (5)$$

where

$$p_n = \frac{c_1\lambda_1\alpha_1^2}{K_n},\ q_n = \frac{c_3\lambda_1\bar{\alpha}_1^2}{K_n},\ K_n = \omega_1^2 + c_2\lambda_1|\alpha_1|^2 - n^2\omega^2 - \frac{\lambda_2\lambda_3}{\omega_2^2 - n^2\omega^2}. \qquad (5a)$$

For non-zero α_n's, the following necessary and sufficient condition in infinite determinant form must be satisfied:

$$\Delta \equiv \begin{Vmatrix} \cdots & \cdots & \cdots & \cdots & \cdots & \cdots & \cdots & \cdots & \cdots \\ \cdots & 1 & 0 & q_{-3} & 0 & 0 & 0 & 0 & \cdots \\ \cdots & 0 & 1 & 0 & q_{-2} & 0 & 0 & 0 & \cdots \\ \cdots & p_{-1} & 0 & 1 & 0 & q_{-1} & 0 & 0 & \cdots \\ \cdots & 0 & p_0 & 0 & 1 & 0 & q_0 & 0 & \cdots \\ \cdots & 0 & 0 & p_1 & 0 & 1 & 0 & q_1 & \cdots \\ \cdots & 0 & 0 & 0 & p_2 & 0 & 1 & 0 & \cdots \\ \cdots & 0 & 0 & 0 & 0 & p_3 & 0 & 1 & \cdots \\ \cdots & \cdots & \cdots & \cdots & \cdots & \cdots & \cdots & \cdots & \cdots \end{Vmatrix} = 0 \qquad (6)$$

The "principal frequency" ω can be determined as a root of the "frequency equation" (6).

To calculate the coefficients α_n, in the case α_1 dominant, we see that by using the notation,

$$u_n = \frac{\alpha_n}{\alpha_{n-2}}, \qquad (7)$$

the recursion formula (5) gives

$$u_n = -\frac{p_n}{1 + q_n u_{n+2}}$$

which leads to the infinite continued fraction,

$$\alpha_n/\alpha_{n-2} = -\cfrac{p_n}{1 - \cfrac{p_{n+2}q_n}{1 - \cfrac{p_{n+4}q_{n+2}}{1 - \ldots}}} \tag{8}$$

The infinite continued fraction (8) can be expressed by means of infinite determinants, and the coefficients α_n can be calculated in terms of the arbitrary α_0 and α_1

Having now calculated the principal frequency ω and the coefficients α_n, the solution (3), by using (4ii), reads:

$$x(t) = \sum_{n=-\infty}^{\infty} \alpha_n e^{in\omega t}, \quad y(t) = \sum_{n=-\infty}^{\infty} \frac{\lambda_3}{\omega_2^2 - n^2\omega^2}\, \alpha_n e^{in\omega t}. \tag{9}$$

The principal frequency ω is the same for both oscillators, and since the coefficients of (9) satisfy the condition (1), then (9) gives the "principal modes" of our nonlinear system.

The method followed is based on the use of exponential series with complex coefficients leading to infinite determinants when one calculates either the frequency or the coefficients of the series. It is similar to the discussion given by L. Brillouin for Hill and Mathieu functions.[2] Next will be discussed the infinite determinants involved in the present note and the solution in its final form given. It may be mentioned that Hill, in his "Lunar Theory," brought into notice the infinite determinants, and Poincaré first gave conditions of their convergence.

The author is indebted to Dr. G. Nomicos for a comment on a point of the note.

[1] Rosenberg, R., ASME, Appl. Mech. Div. paper No. 59-A-93.

[2] Brillouin, L., *Wave Propagation in Periodic Structures*, 2nd ed. (New York: Dover Publications, Inc., 1953).

INVESTIGATIONS ON FINITE GROUPS

BY MICHIO SUZUKI

DEPARTMENT OF MATHEMATICS, UNIVERSITY OF ILLINOIS

Communicated by Richard Brauer, October 10, 1960

1. The primary purpose of this investigation is to determine the structure of finite groups which satisfy the following condition

(*CN*): the centralizer of any nonidentity element is nilpotent.

We call a group G a (*CN*)-group if G satisfies the condition (*CN*). In general, a

(P)-group is a group satisfying a group theoretical condition (P). The results of this note will clarify the structure of (CN)-groups of finite order.

It turns out that there is a class of finite groups which is closely related to the class of (CN)-groups. We consider the condition

(CIT): G is a finite group of even order in which the centralizer of every involution is a 2-group.

Thus, a (CIT)-group is a finite group of even order which contains no element of order $2p$ with $p > 2$, vice versa.

There is no apparent connection between these two classes of groups, but the following theorem is proved.

THEOREM 1. A nonsolvable (CN)-group of finite order is a (CIT)-group.

This is by no means trivial. Many recent results by Brauer, Feit, M. Hall, Thompson, and the author must be used.[1-3] The author has been unable to decide whether the converse statement is true (under the assumption of nonsolvability). So far, all the known nonsolvable (CIT)-groups are (CN)-groups. Anyway, the study of nonsolvable (CN)-groups is reduced to the study of (CIT)-groups, which are rather easy to handle.

Another class of finite groups attracts our attention. Zassenhaus,[8] has studied a finite group G which satisfies the condition

(Z): G is faithfully represented as a doubly transitive permutation group and, in this representation, no element except the identity leaves three distinct letters invariant.

If the representation here is of degree N, then N is called the degree of the (Z)-group G. Recently, Feit[2] has proved that if a (Z)-group G does not contain a normal Frobenius group, then the degree N is of the form $1 + q$ where q is a power of a prime number. We shall denote by (ZT) the following condition on G.

(ZT): G is a (Z)-group of odd degree and G is not a Frobenius group.

It is not difficult to prove the following result.

THEOREM 2. A (ZT)-group is a simple (CIT)-group of composite order.

The linear fractional groups $LF(2,2^n)$ $(n > 1)$ are examples of (ZT)-groups, which were in fact the only (ZT)-groups known until quite recently. Elsewhere, the author[6] has given another type of (ZT)-group $G(q)$ $(q = 2^{2n+1};\ n > 1)$. Besides these two infinite series, no other (ZT)-group is known so far.

The main result of this investigation is that, roughly speaking, the class of (ZT)-groups is almost equal to the class of simple (CIT)-groups. More precisely, we have

THEOREM 3. A simple (CIT)-group is either a (ZT)-group or one of the classical linear fractional groups.

It is easily shown that there is a very small number of classical linear groups which satisfy the condition (CIT). If p is either a Fermat prime or a Mersenne prime, then the linear fractional group $LF(2,p)$ is a (CIT)-group. Besides this class of groups, we have only two other groups, namely, $LF(2,9)$ and $LF(3,4)$, in this category.

The proof of Theorem 3 is very long and difficult, and cannot be discussed in this short note. We just mention that an idea of Thompson[3,7] is used with great advantage and the theory of characters is needed together with an idea similar to the one in ref. 5.

2. The structure of solvable (CIT)-groups is fairly easy to discuss.

THEOREM 4. Let G be a (CIT)-group. If G contains a proper normal subgroup of odd order, then G is solvable. In this case, G contains an abelian normal subgroup A of odd order such that $G = AS$ for a Sylow 2-group S of G and no element $\neq 1$ of S commutes with an element $\neq 1$ of A. If G is a solvable (CIT)-group and if G contains no proper normal subgroup of odd order, G has a series

$$G \supset L \supset N \supset \{1\}$$

of normal subgroups L and N such that G/L and L/N are cyclic groups of relatively prime orders, N is a 2-group and G is a splitting extension of N by G/N. If, moreover, the order of G/N is even, G/L is a 2-group and induces fixed-point-free automorphisms of L/N.

This structure theorem on solvable (CIT)-groups plays a prominent role in the proof of Theorem 3. As for the structure of general nonsolvable (CIT)-groups, we have the following proposition.

THEOREM 5. Let G be a nonsolvable (CIT)-group and N be the maximal solvable normal subgroup of G. Then, N is a 2-group. The quotient group G/N is a simple (CIT)-group with one exception. In the exceptional case, the group G/N is the group, of order 720, which is M_9 in the notation of Zassenhaus.[8]

The group M_9 is the projective group over the near-field of 9 elements. M_9 contains the group $LF(2,9)$ as a normal subgroup of index 2.

3. In proving the first theorem, we need a theorem on a characterization of (ZT)-groups. For an element σ of a group G, we denote by $C_G^*(\sigma)$ the set of elements of G which transform σ into either σ or σ^{-1}. Then $C_G^*(\sigma)$ is a subgroup of G which contains the centralizer $C_G(\sigma)$ of σ as a normal subgroup of index $\leqq 2$. If H is a subgroup of G, we denote by H_0 the subgroup of H generated by involutions in H. If the order of H is odd, we simply set $H_0 = \{1\}$. We have the following result.

THEOREM 6. Let G be a finite group. Assume that G contains a subgroup H satisfying the conditions (1) for any $\sigma \neq 1$ of H we have $C_G^*(\sigma) \subseteq H$, and (2) the center of H_0 is not trivial. Then, we have one of the following four cases: (i) H_0 is a normal subgroup of G, (ii) a Sylow 2-group of G is cyclic, (iii) a Sylow 2-group of G is a generalized quaternion group, or (iv) G is a (ZT)-group.

If the order of G is more than 2, then in the first three cases we have a proper normal subgroup of G. For the case (i), this remark is trivial. Burnside's famous theorem may be applied in the second case. The nonsimplicity of G in the third case has been proved by Brauer and the author.[1] Thus, Theorem 6 gives a characterization of (ZT)-groups among simple groups of composite order. Condition (1) of Theorem 6 can be stated in another (weaker) form. But we shall not go into details here.

As a special case of Theorem 6, we obtain

THEOREM 7. If G contains a nilpotent subgroup H of even order such that $H \supseteq C_G(\sigma)$ for any $\sigma \neq 1$ of H, then the same conclusion as in Theorem 6 holds.

Here, the assumption of nilpotency for H can be replaced by a weaker one. Assuming that H is abelian, Wall and the author[4] have obtained a similar theorem. The proof of these generalizations utilizes similar ideas used by Wall and the author and by Feit[2], who has given another generalization of the abelian case. Theorem 7 is applied to prove Theorem 1 together with other theorems mentioned before.

* This investigation is supported by the National Science Foundation G. 9654.

[1] Brauer, R., and M. Suzuki, "On Finite Groups of Even Order Whose 2-Sylow Group is a Quaternion Group," these PROCEEDINGS, **45**, 1757–1759 (1959).

[2] Feit, W., "On Groups Which Contain Frobenius Groups as Subgroups," *Proc. Symp. Pure Math.*, **1**, 22–28 (1959).

[3] Feit, W., M. Hall, and J. Thompson, "Finite Groups in Which the Centralizer of Any Non-identity Element Is Nilpotent," (to appear).

[4] Suzuki, M., "On Characterizations of Linear Groups, I," *Trans. Amer. Math. Soc.*, **92**, 191–204 (1959).

[5] Suzuki, M., "On Characterizations of Linear Groups, II," *Trans. Amer. Math. Soc.*, **92**, 205–219 (1959).

[6] Suzuki, M., "A New Type of Simple Groups of Finite Order," these PROCEEDINGS, **46**, 868–870 (1960).

[7] Thompson, J., Thesis, University of Chicago (1959).

[8] Zassenhaus, H., "Kennzeichnung endlicher linearer Gruppen als Permutationsgruppen," *Abh. aus dem Math. Seminar der Hamb. Univ.*, **11**, 17–40 (1936).

---

*CONVERGENCE IN NORM**

BY G. T. WHYBURN

UNIVERSITY OF VIRGINIA

Communicated October 12, 1960

1. *Introduction.*—By the *norm* $N(f)$ of a mapping $f(X) = Y$ is meant the least upper bound of $\delta[f^{-1}(y)]$ for $y \in Y$. Here X and Y are metric spaces and $\delta(K)$ is the diameter of K for any set K. We shall be concerned with sequences of mappings (f_n) which not only converge in the usual way to a mapping f, but also converge in norm in the sense that $N(f_n) \to 0$ as $n \to \infty$.

A sequence of sets Y_n is said to converge 0-regularly to a set Y [written $Y_n \to Y$ 0-regularly] provided that $Y_n \to Y$ in the sense lim sup Y_n = lim inf Y_n = Y and also for any $\epsilon > 0$ a $\delta > 0$ and an integer N exist such that if $n > N$, any two points $x, y \in Y_n$ with $\rho(x, y) < \delta$ lie together in a connected subset of Y_n of diameter $< \epsilon$. All spaces are assumed to be metric; and for any open set U in a space, $Fr(U)$ denotes the boundary $\bar{U} - U$ of U. Also, Φ denotes the empty set.

2. THEOREM. *Let the sequence of closed mappings $f_n(X) = Y_n \subset Z$ converge almost uniformly to a mapping $f(X) = Y$, where X is locally compact and $Y_n \to Y$ 0-regularly. Then if $N(f_n) \to 0$ with $1/n$, f is quasi open, and if $f^{-1}(y)$ has a non-empty compact component for each $y \in Y$, f is compact and monotone.*

This will be a direct result of the following.

LEMMA. *For any compact component K of $f^{-1}(y)$, for $y \in Y$, and any open set V in X containing K, there exists a compact set H with $K \subset \text{int } H \subset H \subset V$, an integer N and an open set R in Z about y such that*

$$f_n(X - H) \cdot R = \Phi, \text{ for all } n > N.$$

To obtain the theorem from this lemma we note first that $f(X - H) \cdot R$ must also be empty by convergence of f_n to f, so that

$$f(V) \supset f(H) \supset R \cdot Y$$

and f is quasi-open. Further, since V is an arbitrary neighborhood of K and we have $f^{-1}(y) \subset V$, this gives $f^{-1}(y) = K$. Thus f is monotone if each $f^{-1}(y)$ has a compact component; and since any quasi-open monotone mapping is compact, f must also be compact under these conditions.

Now to prove the lemma, let U_1 be an open set in X containing K with $\bar{U}_1$ compact, $\bar{U}_1 \subset V$ and $Fr(U_1) \cdot f^{-1}(y) = \Phi$. Then take an open set $U_2 \supset \bar{U}_1$ so that $H = \bar{U}_2$ is compact, $H \subset V$ and $(H - U_1) \cdot f^{-1}(y) = \Phi$. Since $N(f_n) \to 0$ and $\rho(\bar{U}_1, X - U_2) > 0$, there exists an integer N_1 such that

(i) $$f_n(\bar{U}_1) \cdot f_n(X - U_2) = \Phi \text{ for all } n > N_1.$$

Now let $6\epsilon = \rho[y, f(H - U_1)]$. Then by uniform convergence of f_n to f on the compact set $H - U_1$, there exists an integer N_2 such that

(ii) $$f_n(H - U_1) \cdot V_{3\epsilon}(y) = \Phi \text{ for all } n > N_2.$$

By regular convergence there exists a $\delta > 0$, $\delta < \epsilon$, and an integer N_3 such that any two points $p, q, \epsilon\, Y_n$ with $\rho(p,q) < \delta$ and in $n > N_3$ lie together in a connected subset C of Y_n with $\delta(C) < \epsilon$. Now let N_4 be chosen so that $f_n(K) \cdot V_{\delta/2}(y) \neq \Phi$ for $n > N_4$. Finally, we define $N = N_1 + N_2 + N_3 + N_4$ and $R = V_{\delta/2}(y)$.

We must show that $f_n(X - H) \cdot R = \Phi$ for $n > N$. Suppose, on the contrary, that this set contains a point q for some fixed $n > N$. Then, since $n > N_4$, $f_n(K) \cdot R$ contains a point p. However, $\rho(p,q) < \delta$, and since $n > N_3$, there exists a connected subset C of Y_n containing $p + q$ and with $\delta(C) < \epsilon$. Also, $C \subset V_{3\epsilon}(y)$, because $\rho(p,y) < \epsilon$; and thus $C \cdot f_n(H - U_1) = \Phi$ by (ii). This gives

$$C \subset f_n(\bar{U}_1) + f_n(X - U_2),$$

with C intersecting the sets on the right in at least p and q respectively. However each of these sets is closed by closedness of f_n, and their intersection is empty by (i). This contradicts the connectedness of C. Thus the lemma is established.

COROLLARY. *Let the sequence of mappings $f_n(X) = Y$ converge uniformly to the mapping $f(X) = Y$ where X is compact and Y is locally connected. Then if $N(f_n) \to 0$ with $1/n$, f is monotone.*

For, in this case, Y is a locally connected continuum and $Y_n = Y$, so that the 0-regularity condition is automatically satisfied.

3. THEOREM. *Let the sequence of mappings $f_n: X \to Z$ converge (pointwise) to the mapping $f(X) = Y$, where X is a locally connected generalized continuum and suppose $N(f_n) \to 0$ with $1/n$. Then if X is cyclic, any two points of Y are conjugate in Z.*

NOTE. A connected set is *cyclic* if it has no cut point; and two points a and b in a space Z are *conjugate* provided they cannot be separated in Z by any single point of Z.

To prove the theorem, we suppose, on the contrary, that some point y in Z separates two points of Y in Z so that we have a separation

$$Z - y = Z_1 + Z_2$$

where $Y \cdot Z_1 \neq \Phi \neq Y \cdot Z_2$. Let $A = Y \cdot Z_1$, $B = Y \cdot Z_2$. Our conclusion follows at once from the two assertions:

(i) If $L = \limsup f_n^{-1}(y)$, then $L \neq \Phi$.

(ii) Any $p \in L$ is a cut point of X.

Now to verify (i), we have only to take $a \in A$, $b \in B$, $a' \in f^{-1}(a)$, $b' \in f^{-1}(b)$ and let $a'b'$ be any simple arc in X from a' to b'. Then, since $f_n(a') \to a$ and $f_n(b') \to b$, we have $f_n(a') \in Z_1$, $f_n(b') \in Z_2$ for all n sufficiently large. Thus $f_n(a'b') \supset y$ and $a'b' \cdot f_n^{-1}(y') \neq \Phi$ for all such n, so that $L \cdot a'b' \neq \Phi$.

To show (ii), let $p \in L$ and let $a \in A$ and $b \in B$ be chosen so that $a \neq f(p) \neq b$. Then if $a' \in f^{-1}(a)$, $b' \in f^{-1}(b)$, every arc $a'b'$ in Z intersects almost all the sets $f_n^{-1}(y)$ as just shown in the preceding paragraph. However, since $\delta[f_n^{-1}(y)] \to 0$ with $1/n$ this gives $p \in a'b'$. Thus, p lies on every arc in Z from a' to b', so that p separates a' and b' in Z.

COROLLARY. *Let the sequence of mappings $f_n(X) = Y$ converge (pointwise) to the mapping $f(X) = Y$, where X and Y are locally connected generalized continua, and suppose $N(f_n) \to 0$ with $1/n$. Then, if X is cyclic, so also is Y.*

4. *Application to mappings on the 2-sphere.*—It now follows that if X and Y are locally connected continua, with X cyclic, and $f_n(X) = Y$ is a sequence of mappings converging uniformly to $f(X) = Y$ and with $N(f_n) \to 0$, then f is monotone and Y is cyclic. In this form the result is of especial interest in case the domain space X is the 2-sphere. For it then follows that Y is cyclic and also is the monotone image of the 2-sphere so that it must itself be a (topological) 2-sphere. Hence Y is homeomorphic with X in this case.

It should be noted, however, that the limit mapping $f(X) = Y$ need not be a homeomorphism even in this case. For we can easily define a sequence of homeomorphisms of a sphere onto itself which converges uniformly to a monotone mapping which is not a homeomorphism. Indeed, by a theorem of Youngs,[1] *every* monotone mapping of the 2-sphere onto itself can be uniformly approximated by homeomorphisms. By our results above, no other type of mapping on the 2-sphere can be uniformly approximated by onto mappings of any sort with norms converging to 0. Thus if $f_n(X) = Y$ converges uniformly to $f(X) = Y$ and $N(f_n) \to 0$, where X is a 2-sphere, for any $\epsilon > 0$ there exists a homeomorphism $h(X) = Y$ with $\rho(f_n,h) < \epsilon$ for almost all n.

5. *Uniform openness.*—A sequence of mappings $f_n(X) = Y$ is uniformly approximately open[2] provided that for any $\epsilon > 0$ a $\delta > 0$ and an integer N exist such that for any $x \in X$ and any $n > N$, $f_n[V\epsilon(x)] \supset V_\delta[f_n(x)]$. Clearly, this is equivalent to the condition that for any $\epsilon > 0$ a $\delta > 0$ and integer N exist such that for x, $y \in Y$, $\rho(x,y) < \delta$ and $n > N$ imply that

$$f_n^{-1}(x) \subset V\epsilon[f_n^{-1}(y)] \text{ and (automatically) } f_n^{-1}(y) \subset V\epsilon[f_n^{-1}(x)].$$

Now if X and Y are compact and the sequence $f_n(X) = Y$ converges uniformly to the mapping $f(X) = Y$, uniform openness implies at once that for any $\epsilon > 0$ we have

$$f^{-1}(y) \subset V\epsilon[f_n^{-1}(y)] \text{ and } f_n^{-1}(y) \subset V\epsilon[f^{-1}(y)]$$

for all $y \in Y$ and n sufficiently large. Thus we have $\lim_{n\to\infty} f_n^{-1}(y) = f^{-1}(y)$ for each $y \in Y$. Compare this form with Polak[3]). Hence we have immediately the

THEOREM. *Let the sequence $f_n(X) = Y$ converge uniformly to $f(X) = Y$ where X and Y are compact and suppose the sequence (f_n) is uniformly approximately open. Then if $N(f_n) \to 0$ with $1/n$, f is a homeomorphism.*

This theorem together with an earlier result of the author[2] gives the

COROLLARY. *Let the sequence of homeomorphisms $h_n(X) = Y$ converge uniformly to a mapping $h(X) = Y$. In order that h be a homeomorphism it is necessary and sufficient that the sequence (h_n) be uniformly approximately open.*

* This research was supported by the United States Air Force through the Air Force Office of Scientific Research of the Air Research and Development Command, under Contract AF 49(638)-72 at the University of Virginia.

[1] Youngs, J. W. T., "Homeomorphic approximations to monotone mappings," *Duke Math. Jour.*, **15**, 82–94 (1948).

[2] Whyburn, G. T., "Sequence approximations to interior mappings," *Am. Soc. Polon. Math.*, **21**, 147–152 (1948).

[3] Polak, A. I., "On mechanisms of uniform approximations in the domain of continuous mappings of compacta" (Russian), *Uspekhi Mat. Nauk (NS)*, **11**, No. 4 (70), 149–154 (1956).

---

*SIGNIFICANCE OF CONTINUED VIRUS PRODUCTION IN TISSUE CULTURES RENDERED NEOPLASTIC BY POLYOMA VIRUS**

BY RENATO DULBECCO AND MARGUERITE VOGT

CALIFORNIA INSTITUTE OF TECHNOLOGY

Communicated by G. W. Beadle, October 24, 1960

The infection with polyoma (PY) virus of monolayer cultures of either mouse or hamster embryo cells gives rise to a neoplastic transformation of the cultures.[1] Previous results showed that the transformed hamster cultures did not release any virus, whereas the transformed mouse cultures continued to release PY virus indefinitely; in the latter case, the virus released was invariably a mutant of small-plaque (*sp*) type.[2] We have since observed that in some cases transformed hamster cultures also continue to release virus indefinitely and that the virus released is also of the *sp* type.

These observations raise a number of questions: Why do transformed cultures release virus indefinitely in some cases and not in others? Why is the released virus of these cultures, once transformed, invariably the *sp* mutant? How is the release of virus related to the transformation?

To try to answer these questions, we have studied in detail the evolution of the infected cultures, the properties of the transformed cells, and the quantitative aspects of virus release from transformed mouse embryo cultures.

Results.—Growth characteristics of hamster and mouse embryo cultures transformed by PY virus: One of the main *in vitro* characteristics of transformed mouse or hamster embryo cultures is an increased "over-all growth rate," as compared to that of noninfected cultures. A measure for the "over-all growth rate," i.e. the average rate at which a culture grows when maintained by serial transfer, was obtained as follows. All the cultures were grown as monolayers in contact with the bottom of petri dishes and were transferred without delay as soon as their cells had grown into a confluent sheet. At each transfer, a known fraction of the trypsin-dispersed culture was used as the inoculum. Thus, the increment in cellular mass at every transfer was known, as well as the time required to achieve it. Plotting the logarithm of the product of all increments at successive transfers versus

the time at which the transfers were carried out supplies the "over-all growth curve" of the culture. The characteristics of the curve depend on many factors, such as the dependence of the multiplication rate of the cells on the degree of crowding of the culture, the proportion of cells surviving the transfer, the length of the lag period after transfer, etc. Cell killing by virus is not an important factor except in mouse embryo cultures during the first month following infection when cell degeneration is extensive.

Over-all growth curves for a number of cultures of hamster embryo cells infected independently with PY virus are given in Figure 1. All curves tend to a final

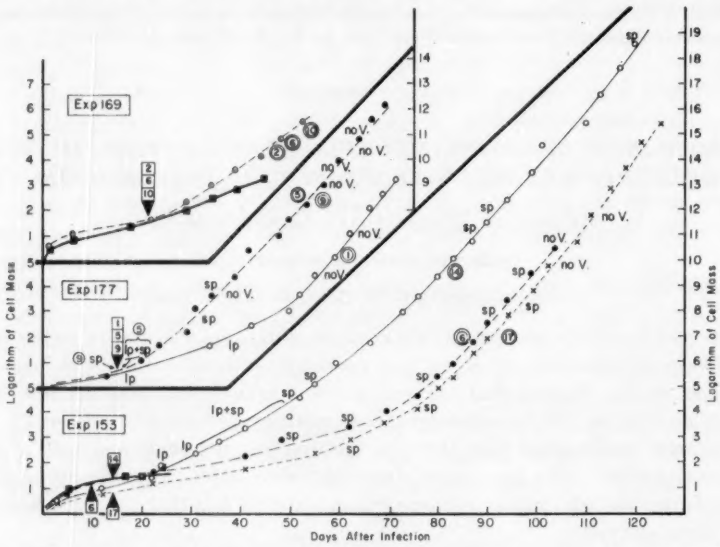


FIG. 1.—Over-all growth curve of hamster embryo cultures infected with PY virus. The heavy lines give the cell mass of uninfected cultures, the unit being the mass of a confluent sheet of cells; the thin lines give the mass of infected cultures, in the same units. The numbers in the double circles are those of the respective cultures. A large black triangle indicates the time of appearance of whorls and excessive acidification in the culture designated by the number associated with it. The designations *lp*, *sp* indicate the type of virus produced by the cultures at the corresponding time; *no V* indicates that no infectious virus was produced.

Cultures 153-14, 177-1, and 169-2 were infected with virus of *lp* type; cultures 153-6, 153-17, 177-9, and 169-10, with virus of *sp*-type; cultures 177-5 and 169-6, with an equal mixture of the two types.

increased growth rate which is approximately the same for all cultures. The increase in the growth rate occurs after the first changes observable in the culture, which consist in whorl formation and increased acidification.[1] Thus, three stages can be identified in infected hamster embryo cultures: pre-whorl, whorl, and enhanced growth rate. It is likely that the transition between the second and third stage occurs when the cells constituting the whorls—which must have an over-all growth rate greater than that of normal cells and which have been shown to be neoplastic[1]—take over the whole culture. The variability in the time required for this transition is due mostly to the technical difficulty of dispersing the whorls by trypsin.

Over-all growth curves of mouse embryo cultures given in Figure 2 also show at the final stage an increase in growth rate, probably of similar significance.

Time and mode of appearance of sp virus in cultures infected by large-plaque (lp) virus: As shown in Figure 1, most hamster embryo cultures cease releasing virus when they reach the stage of enhanced growth rate; cultures infected with *sp* virus tend to release virus for a somewhat longer time than cultures infected with *lp* virus. An exception is constituted by culture 153–14; this culture was infected with *lp* virus and continued to release *lp*-type virus during the first and second stage but changed to release of *sp*-type virus in the third stage.

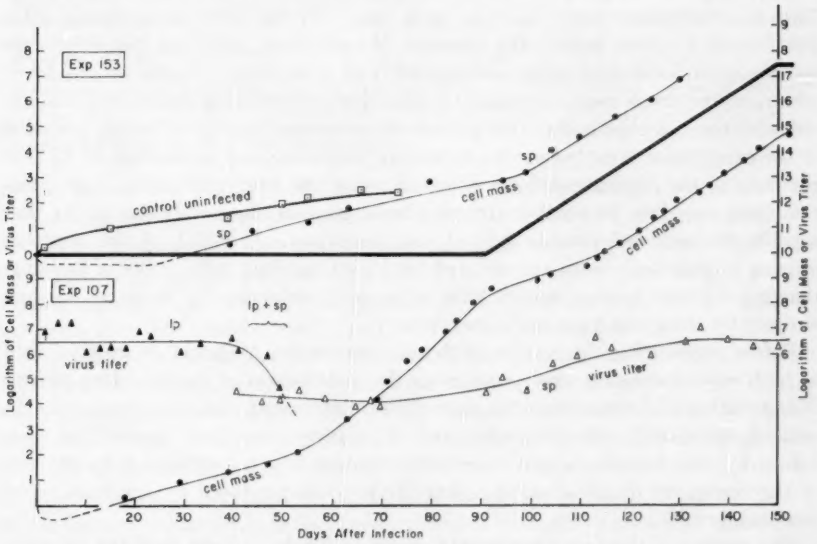


FIG. 2. Over-all growth curves of mouse embryo cultures infected with PY virus. The symbols are the same as those of figure 1. In addition, in the lower curve, ▲ = titer of *lp* virus present in the culture; Δ = titer of *sp* virus. Titers are given as PFU per culture.

The culture of experiment 107 had been infected with virus of *lp*-type; that of experiment 153 with virus of *sp*-type. The temporary decrease in growth rate of culture 107 around the 100th day was due to accidental causes.

Figure 2 shows that the infected mouse embryo cultures released virus all the time. Culture 107, which had been infected with *lp* virus, released *lp* virus until its over-all growth rate increased, and changed then, like the hamster embryo culture 153–14, to release of *sp* virus. This change in the type of virus released appears to be the usual one in mouse embryo cultures infected with *lp* virus.

These observations concerning the continued release of *sp* virus were made on the mass cultures infected by PY virus. In an attempt to evaluate its significance, virus release was studied at the level of the individual cells in the transformed, virus-releasing mouse embryo culture 107. Two questions were asked: Do all the cells of the cultures have the property of virus release? Is this property transmitted hereditarily? The two following groups of experiments answer these questions.

The distribution of the virus and of the virus yields in the cells of the virus-releasing mouse embryo line 146: To answer the first question, two experiments were carried out by using two cultures of line 146 (a derivative of line 107) showing persistent release of *sp* virus; one was an untreated culture, the other had been exposed for 35 days to an antiviral hamster serum of medium potency. The treatment with antiserum, on the basis of preliminary experiments, was expected to decrease the amount of infectious virus adsorbed to the cells.

In each case, the culture was dispersed by trypsin; the cells of the resulting cell suspension were distributed, under a binocular microscope,[3] into drops of medium kept under paraffin oil. Exactly ten cells were placed into each drop. Two determinations were made in each case. In the first, immediately after distribution (= zero point), the contents of each drop, including the cells, were transferred to individual tubes containing 0.5 ml of medium. In the second determination, the drops were incubated for four days before being transferred to individual tubes. At the end of this period, there was an average of 8 cells per drop in the experiment done before the antiserum treatment and an average of 45 cells per drop in the experiment done after the antiserum treatment, with little variation from one drop to another (for accidental reasons, the conditions in the first experiment were unfavorable for cell multiplication). One-fifth of the contents of each tube in both tests was assayed for PY virus after three cycles of freezing-thawing. Tubes having no or little virus were subsequently assayed in their entirety by using 3 to 4 plates for each tube.

Before considering the results of these experiments, it should be recalled that in both experiments the zero point gives the distribution of mature virus present in association with the cells at the start of the experiment; this virus represents the sum of superficially adsorbed virus and of progeny virus that has not yet been released. The four-day count gives a distribution which corresponds to the sum of the zero-point distribution plus that of the virus produced by the cells in the four-day period.

The results of the two experiments, given in Table 1, show that the four-day distributions can be divided into two parts (which are separated by a vertical line in the table). In each case, the left part is equivalent to the zero-point distribution; the right part is characterized by larger amounts of virus in the drops. Since these larger amounts are absent in the zero-point distributions, most of them must represent yields of progeny virus produced during the four-day period. The right parts of the four-day distributions are similar in the experiments before and after antiserum; in contrast, the zero point distribution and the left part of the four-day distribution show a great reduction in the amount of virus in the antiserum-treated culture (compare, for instance, the zero classes). This result suggests very strongly that the zero-point distributions and the left part of the four-day distributions mostly represent virus superficially adsorbed to the cells.

The data of Table 1 show that larger yields were present in 12 out of 75 drops collected after four days in the experiment before the antiserum treatment, and in 32 out of 95 drops collected after four days in the experiment after antiserum. Since the large yields were present in a minority of the drops, most of them must have been produced by single cells. Therefore, as a first approximation, 12 out of 750 (1.6%) initial cells yielded progeny virus in the experiment before antiserum,

TABLE 1

DISTRIBUTION OF VIRUS IN DROPS OF MEDIUM SEEDED WITH TEN CELLS OF LINE 146

Experimental point	Plaque-forming units per drop																Number of drops
	0	1–10	11–50	51–100	101–150	151–200	201–250	251–300	301–350	351–400	401–450	451–500	501–550	551–600	601–650	651–700	>700	
Before antiserum																		
Zero point	0	10	9	4	2	..	..	..	..	..	..	..	..	..	..	..	..	25
4-day determination	1	35	15	10	2	3	4	..	2	..	..	..	..	..	1	..	2	75
After antiserum																		
Zero point	31	14	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	45
4-day determination	50	13	5	9	5	1	4	..	3	2	..	..	2	..	..	..	1	95

In the experiment before antiserum, 4740 PFU of progeny virus (right part of the 4-day distribution) were produced by 12 cells, with an average yield of 395 PFU per cell; if calculated on the basis of the 750 cells used in the experiment, there was an average virus production of 1.6 PFU per cell per day. In the experiment after antiserum, 6614 PFU were produced by 32 cells, with an average yield of 207 PFU per cell. The average production, on the basis of the 950 initial cells, was 1.7 PFU per cell per day.

and 32 out of 950 (3.3%) initial cells did so in the experiment after antiserum; the average yield per virus-producing cell was 395 and 207 PFU, respectively.

That virus production was normal in the drops is seen by the similarity of the yield per cell, averaged over all the cells, in the drop experiments and in a comparable mass culture. In fact, the average yield was about one PFU per cell per day in the drop experiments (see Table 1) and in the mass culture.

Properties of clones derived from the virus-releasing mouse embryo line 146: To determine whether the property of releasing virus is transmitted hereditarily, two cloning experiments were done. In each experiment, a culture of the subline 146(3), which was kept under antiviral serum and on which the distribution of the previous section was carried out, was dispersed with trypsin; single cells were transferred under microscopic observation to petri dishes containing as feeder layers secondary mouse embryo cultures which had been irradiated with 5000 r on the previous day. To avoid viral or cellular cross-contamination of the clones, only one cell was transferred to each feeder plate. In all, 200 cells were distributed. Of these, 43 were lost due to contamination or detachment of the cell layer; of the remaining 157 cells, 82 developed into clones; the cloning efficiency was therefore 56 per cent. Each clone gave rise to an actively growing culture.

The clonal cultures were kept under observation for one month in experiment 192 and for three months in experiment 198; during this period they were repeatedly tested for the presence of PY virus. It was found that 53 per cent of the clones in experiment 192 and 51 per cent in experiment 198 never released virus, whereas the remaining cultures released virus at a rate comparable to that of the parental culture. The observed proportion of clones not releasing virus is a minimal value, since clones not releasing virus could have become superinfected (see later) as a consequence of virus multiplication in the feeder layer; in fact, infection of the susceptible feeder layer by virus introduced with the cell is to be expected in some cases. From the point of view of morphology or growth characteristics, the clonal cultures not-releasing virus could not be differentiated from the cultures releasing virus. Thus, although the transformed mouse embryo culture obtained after infection with PY virus and kept as mass culture in the presence of antiviral serum

for a month continued to release virus, it contained many cells which on cloning gave rise to cultures not releasing virus.

To test whether the simultaneous presence, in the mass culture, of cells releasing virus and of cells giving rise to clones not releasing virus was a consequence of a heterogeneity of the culture before infection, a clonal culture releasing virus was in turn cloned after a brief exposure to antiviral serum. Again, the majority of these second-generation clones did not release virus, thus showing that the difference in the properties of the cells to release virus are not due to a preexisting heterogeneity.

The isolation of clones not releasing virus from the virus-releasing mass culture explains the finding that tumors induced in mice by PY virus may cease to produce virus upon transplantation.[4, 5]

Superinfectibility by PY virus of clonal cultures not releasing virus: The results described in the previous sections show that the majority of the cells in the virus-releasing mass culture do not release virus and do not transmit the potentiality for spontaneous virus release to their progeny. This raised the question of whether the persistent virus release in the mass culture was due to the continued reinfection of a small proportion of the cells.

Superinfection experiments were therefore carried out with five of the clonal mouse embryo cultures not releasing virus from experiment 198. Subcultures of these clonal lines, when infected with either *sp* or *lp* virus became persistent virus-releasers, the amount of virus released being similar to that of the parental mass culture. The study of the distribution of virus yields per cell over a 4-day period, carried out in a drop experiment similar to the one described in a previous section, showed again that only a few per cent of the cells became virus yielders. Furthermore, the cultures which had been superinfected with *lp* virus became releasers of *sp* virus 20 to 30 days after superinfection. Thus the superinfected clonal cultures became indistinguishable from the parental virus-releasing mass culture.

It should be remarked that the clonal cultures are considerably more resistant to PY virus than normal mouse embryo cultures. In fact, when superinfected, they show not only a small proportion of cells releasing virus, but also an unaltered overall growth rate and the absence of noticeable cell degeneration.

Conclusions.—The present results show that the persistent virus release, which is present in some of the cultures transformed by PY virus and absent in others, is a superimposed phenomenon. The neoplastic cells arising as a consequence of virus infection in either mouse or hamster embryo cultures are intrinsically nonvirus-releasers. Persistent virus release is caused and maintained in the mass cultures by reinfection of a small proportion of the cells. In this persistent infection, the *sp* mutant of PY virus, presumably preexisting in *lp* stocks, ultimately predominates; this mutant, which is more cytocidal than the *lp*-type virus on normal sensitive mouse embryo cells,[6] has a selective advantage in cultures of neoplastic cells.

An essential factor in the phenomenon of persistent virus release is the increased resistance of the neoplastic cells to superinfection by PY virus. In most transformed hamster cultures, the resistance is so high that no or very few virus-releasing cells arise upon superinfection; in transformed mouse embryo cultures, the resistance is weaker, and a few per cent of virus-yielding cells can be produced. This small proportion of virus-yielding cells is sufficient to maintain virus release in-

definitely in a state comparable to the "carrier state" obtained in many other animal cell-virus systems.[7, 8]

The mechanism by which the persistent release of *sp* virus arises seems completely distinct from the mechanism by which the PY virus causes the neoplastic transformation. For this reason, the mode of production of *sp* virus does not illuminate the mechanism of neoplastic transformation.

* Aided by grants from the American Cancer Society, the U.S. PHS (grant RG 6965), and the National Foundation.

[1] Vogt, M., and R. Dulbecco, these PROCEEDINGS, **46**, 365–370 (1960).

[2] Medina, D., and L. Sachs, *Virology*, **10**, 387–388 (1960).

[3] Lwoff, A., R. Dulbecco, M. Vogt, and M. Lwoff, *Virology*, **1**, 128–139 (1955).

[4] Sachs, L., and M. Fogel, *Virology*, **11**, 722–736 (1960).

[5] Habel, K., Nat. Canc. Inst. Monograph No. 4, 207 (1960).

[6] Gotlieb-Stematsky, T., and S. Leventon, in press.

[7] Vogt, M., and R. Dulbecco, *Virology*, **5**, 425–434 (1958).

[8] Puck, Th. T., and S. J. Cieciura, in *Symposium on Latency and Masking in Viral and Rickettsial Infections*, eds. Duard L. Walker *et al.* (Burgess, 1957).

---

*THE SPARING EFFECT OF RMC POLIOVIRUS ON PRIMARY AMNION CELL CULTURES**

BY MONTO HO

DEPARTMENT OF EPIDEMIOLOGY AND MICROBIOLOGY,
GRADUATE SCHOOL OF PUBLIC HEALTH, UNIVERSITY OF PITTSBURGH

Communicated by John F. Enders, November 4, 1960

In previous communications,[1, 2] one mechanism whereby cell cultures persist in the presence of an ordinarily cytopathogenic virus was described. Viral inhibitory factor (VIF), probably a variety of interferon,[3] was shown to account for the diminished cytopathic effect (CPE) of a Type II MEF_1 poliovirus variant adapted to the chick embryo (RMC virus[1, 4]) in human amnion cultures receiving undiluted inocula as compared to the complete CPE in those that received diluted inocula. However, it was pointed out[2] that VIF could *not* explain all forms of resistance of amnion cultures to RMC virus, such as that of amnion cells of young *in vitro* age.[5] Further studies of certain resistant cultures have shown that not only is initial CPE overcome but their prolonged survival may be favored.

Materials and Methods.—Primary amnion cultures were prepared as previously described[2] except that instead of Enders' medium containing 5 per cent horse serum, 5 per cent beef embryo extract, 45 per cent bovine amniotic fluid, and 45 per cent Hank's balanced salt solution (BSS), Eagle's basal medium in BSS modified to contain 10 per cent horse serum and antibiotics was used. Cells were grown on the glass surface of 16 mm test tubes containing 1 ml medium. RMC virus used in these studies was from a stock chick embryo passage or from an amnion culture passage. The latter was collected from cultures of amnion cells in Roux bottles two days after stock RMC virus at input multiplicity of about 1 was inoculated. Such harvests also contained VIF.[2]

Results.—In routine titrations of RMC virus, it was customary to use amnion cultures of at least two weeks *in vitro* age and to roll them after inoculation in order to elicit viral CPE.[1, 5] Previously,[1] using amnion cultures nourished with Enders' medium under these conditions, stock RMC virus produced complete CPE.[1] However, using cultures prepared under our present conditions, complete CPE of RMC virus was not consistently observed unless chick embryo extract was added.[6] Thus, in Table 1, typical titrations of three specimens employing amnion cultures one month after they were initiated are presented. Although RMC virus from both a stock culture and from amnion passage developed typical CPE in 3–5 days, this was only partial in character. With weekly to biweekly changes of Eagle's medium, all cultures exhibiting characteristics of infection had recovered in about one month and appeared healthier and more confluent than controls. Furthermore, the cells in such cultures gradually changed in morphology, so that compared to the usual polygonal amnion cells, they were longer and more ellipsoidal and hyaline. They arranged themselves frequently in whorls, and the edges of the culture were smoother than uninfected controls. When samples of centrifuged VIF from which infective virus was removed (see Table 1) were inoculated in amnion cultures or when

TABLE 1

PROGRESSION OF CELLULAR CHANGE IN HUMAN AMNION CULTURES INFECTED WITH RMC VIRUS

Source and dilution of inoculum (0.1 ml)		Days of observation 3–5	15	30	60–120
Stock RMC Virus	10^{-0}	1+	±	sO	C
	10^{-1}	2+	±	O	C
	10^{-2}	1+	±	C	C
	10^{-3}	1+	±	sO	C
	10^{-4}	0	P	—	—
Amnion Passage RMC Virus	10^{-0}	0	O	sO	C
	10^{-1}	±	O	O	C
	10^{-2}	±	O	sO	C
	10^{-3}	1+	O	sO	C
	10^{-4}	±	O	P	C
	10^{-5}	0	P	P	PC
	10^{-6}	0	P	P	P
Centrifuged* VIF	10^{-0}	0	P	P	F
	10^{-1}	0	P	P	P
	10^{-2}	0	P	P	PC
	10^{-3}	0	P	P	—
	10^{-4}	0	P	P	F
	10^{-5}	0	P	P	F
No inoculum		0	P	P	P

* Amnion passage material was centrifuged x2 at 104,000 *g* for 90 minutes.
± = definite but minimal viral CPE: 1+ = CPE, cc 25% cells destroyed; 2+ = CPE, cc 50% cells destroyed.
O = confluent sheet of normal polygonal cells; P = cells scattered, granular, and poor; sO = small patches of normal-appearing cells; F = no viable cells visible; C = confluent sheet of "changed" cells.
PC = poor culture with some areas of "changed" cells.
— = not observed.

uninoculated cultures were kept as controls, no initial viral CPE developed and no such cellular changes were evident in most instances. As expected, the culture receiving undiluted amnion passage virus showed no initial CPE due to the presence of VIF,[1, 2] although the progress of this culture corresponded closely to those that showed early viral CPE. Whether the eventual appearance of "changed" cells in two other cultures described in the table which showed no initial CPE is due to undetected viral effect is at present undetermined. A similar cell-sparing effect consisting of repair of cell damage and appearance of cells of altered morphology

comparable to that described has been repeatedly observed after about 10^6 TCD_{50} RMC virus were inoculated in amnion cultures less than two weeks old and incubated in a roller drum or left stationary.

Some preliminary experiments have been undertaken to elucidate the role of RMC virus in producing this sparing effect.

1. To compare cell multiplication in infected and in control amnion cultures, 20 tube cultures were initiated with a cell suspension containing about 400,000 cells per ml. To each of ten cultures a 0.1 ml inoculum of about 10^6 TCD_{50} RMC stock virus was added, and ten control cultures received 0.1 ml of uninfected chick embryo material. At intervals ranging from two days to two weeks, culture fluids were collected for virus titrations, and the number of cells adhering to the glass surface of each culture was determined by counting in a hemocytometer after they were removed with trypsin. The cell count in both the infected and control cultures was about 45,000 two days after initiation of the cultures and it reached a maximum of about 120,000 in 12 days. After $1^1/_2$ months, however, the inoculated cultures were confluent and healthy in appearance, whereas the cells in the controls were in nonconfluent patches. The cell counts were 70,000 and 50,000 respectively. It appears from a number of such experiments that the presence of virus probably has no accelerating effect on cellular multiplication, but it somehow renders the cells relatively resistant to spontaneous degeneration.

2. To determine the extent and presence of virus in inoculated amnion cultures, representative samples of fluids from cultures used in the experiments described in the above paragraph and in Table 1 were titrated by inoculating 0.1 ml of serial 10X dilutions in amnion cultures, which in parallel experiments were shown to be satisfactory in titrating a stock RMC virus specimen. Similar titrations were carried out in 2–4 day suckling mice, which received 0.02 ml of a dilution intracerebrally. Intracellular virus was released by freezing and thawing a cell suspension 3X. RMC virus was only sporadically detected in these persistent cultures. For example, no virus was detected by using amnion cultures as a test system in the fluid of cultures 1 and 4 months after they had received 10^{-1} stock RMC virus. Parallel tests of fluids from a culture that received 10^{-1} amnion passage virus were similarly negative. However, four months after a culture had received 10^{-2} amnion passage virus, death was produced at 10^{-1} dilution in suckling mice when both fluid and cells were tested together for viral activity. It appears that the altered state of amnion cultures inoculated with RMC virus is accompanied by little if any multiplication of infectious virus. However, the importance of small amounts of virus, which may not always be detectable under the described conditions, is not precluded.

3. To ascertain the role of virus in the fluid phase of these persisting cultures, monkey antiserum against Type II poliovirus previously shown to be sufficient to neutralize infective virus (1:50) was added to two persistent cultures $3^1/_2$ months after they had received RMC virus. Observation for three weeks failed to reveal any difference between these cultures and other persistent cultures without antiserum, showing that the presence of infective virus in the fluid phase is probably not essential for the persistence of the cultures.

Comment.—It appears that when the ordinarily cytocidal RMC virus is added to primary amnion cultures under various conditions, one may observe not only

a spectrum of cell destruction ranging from complete to absent CPE but also a paradoxical sparing effect on the cultures. Further studies are required to ascertain the genetic and nutritional conditions underlying this phenomenon. This sparing effect has some superficial resemblance to that of certain tumor viruses on cell cultures, but there is as yet no evidence that the cells spared have undergone genetic transformation.

Summary.—Primary human amnion cells cultured under defined conditions were inoculated with a variant of Type II poliovirus and observed for over four months. The initial cytopathic effect was overcome, and gradually confluent sheets of healthy-appearing cells of altered appearance developed. In contrast, uninoculated control cultures underwent gradual spontaneous degeneration.

The author is indebted to Drs. John F. Enders, William McD. Hammon and Horace M. Gezon for helpful criticisms.

* This investigation was aided by Grant E-2953 from the National Institutes of Health.

[1] Ho, M., and J. F. Enders, these PROCEEDINGS, **45**, 385 (1959).

[2] Ho, M., and J. F. Enders, *Virology*, **9**, 446 (1959).

[3] Isaacs, A., and J. Lindemann, *Proc. Roy. Soc.*, **147**, 258 (1957).

[4] Kindly supplied by Dr. John F. Enders, who obtained it originally from Dr. H. R. Cox.

[5] Frothingham, T. E., *Proc. Exper. Biol. & Med.*, **100**, 505 (1959).

[6] Unpublished experiments.

---

*REGULATION BY COLIPHAGE LAMBDA OF THE EXPRESSION OF THE CAPACITY TO SYNTHESIZE A SEQUENCE OF HOST ENZYMES**

BY MICHAEL B. YARMOLINSKY AND HERBERT WIESMEYER†

MCCOLLUM-PRATT INSTITUTE, THE JOHNS HOPKINS UNIVERSITY‡

Communicated by Herman M. Kalckar, October 17, 1960

A small group of closely linked genes specifying the enzymes of galactose metabolism is capable of being transduced by the coliphage lambda.[1] A region of the bacterial chromosome or a replica thereof encompassing the transduced genes is inserted into the genome of the transducing phage, replacing a portion of the viral genetic material.[2–4] This relatively stable situation makes possible investigation of the expression of bacterial genes situated in a foreign environment, i.e., within the genome of a prophage. Upon inducing the replication of transducing phage, cells may be obtained prior to lysis, each possessing several copies of a short region of bacterial genetic material. The present work was originally undertaken to investigate the position and gene dosage effects afforded by a study of *Escherichia coli* harboring transducing lambda. Its course has been altered somewhat by an unexpected result obtained in a control experiment using *E. coli* lysogenic for normal lambda alone. Following induction of phage replication, the activity of an enzyme of galactose metabolism was found to increase whether or not the capacity for synthesis of this enzyme, galactose-1-phosphate uridyl transferase, was carried by the phage. Increases in enzyme activity, apparently due to increased dosage of transduced genes, must be interpreted in the light of the increases in

enzyme activity due to induction of normal lambda phage alone. The viral and enzymatic specificity of the latter phenomenon are described here. This newly recognized mode of interaction between phage and host genome offers a new approach to the study of cellular control of metabolic activity. Work of a similar nature, but involving a different enzyme of galactose metabolism, galactokinase, is being pursued by Buttin, Jacob, and Monod.[5]

TERMINOLOGY

The terms of transduction used here are largely as defined by Morse, Lederberg, and Lederberg[6] and incorporate some minor modifications introduced by Arber.[3]

A bacterium susceptible to infection by a particular phage is *sensitive*. Upon infection by a temperate phage, such as lambda, some of the cells are lysed, others lysogenized. A *lysogenized bacterium* harbors the phage as *prophage*, a condition in which each viral genome replicates in association with a bacterial nucleus. In the lysogenized bacterium, the viral functions required for the lytic cycle are under a repression which various inducing agents, such as ultraviolet light or chemical agents, can suspend. Since the viral functions of homologous phages which may enter the cell are also suppressed, the lysogenized cell is said to be *immune*. Phage mutations affecting the specificity of immunity lie within the same locus of the viral genome as mutations affecting the site of prophage localization.[7] Prophage lambda locates adjacent to the complex of bacterial genes governing galactose metabolism, the *gal* region.[23]

The lysogenized bacterium is called an *active lysogen* if the prophage is capable of undergoing its lytic cycle of multiplication, spontaneously or upon induction. The designation *defective phage* (strictly speaking, a contradiction in terms[9]) is applied to a phage whose genome is so altered as to render it incapable of undergoing a complete lytic cycle in single infection, yet capable of conferring immunity upon the cells it lysogenizes. Attempts to induce phage replication in such cells—*defective lysogens*—result in cellular lysis[10] but fail to yield phage progeny or even detectable phage protein.[3] The replication of defective phage is made possible by the simultaneous presence of active phage, so-called *helper phage*. Helper phage serves to make good the deficiency in defective phage by a cooperative process and permits a mixed burst of active and defective phage particles.[2, 3, 11, 12]

Bacteriophage lambda transducing the galactose region of *Escherichia coli* is a defective phage. The functional defect results from deletion of a large region of the phage genome which is replaced by genetic material of bacterial origin. In double lysogens, carrying in addition to a defective transducing prophage an active lambda prophage, the replication of transducing phage, inclusive of transduced genetic material, occurs upon induction.[10] Transductants, being hyperploid for the transduced genetic region, are referred to as *syngenotes: defective syngenotes* if helper prophage is absent, or *lysogenic syngenotes* if helper prophage is present. A syngenote is either a *heterogenote* or a *homogenote* depending upon whether the transduced characters under discussion are genetically different from or identical to those of the recipient. Both defective and lysogenic syngenotes are somewhat unstable and segregate sensitives, predominantly of the original bacterial genotype, with frequencies of the order of $2 \cdot 10^{-3}$ to $2 \cdot 10^{-2}$ per cell division.[1, 3, 6]

An *HFT lysate* is a high-frequency transducing lysate in which the titers of

normal and transducing lambda phage are of the same order of magnitude. HFT lysates are prepared by induction of lysogenic syngenotes. In *LFT* (low-frequency transducing) *lysates*, prepared by induction of active lysogens, the proportion of transducing phages is of the order of 10^{-6} to 10^{-4}.[1, 3, 6] A lysate obtained directly upon infection of a strain sensitive to lambda fails to yield transducing phage.[1] Lysogenic heterogenotes may be selected on indicator agar following treatment of sensitive cells with LFT lysates, while lysogenic homogenotes (and defective syngenotes) are most easily obtained by use of HFT lysates.

The term induction is used here in two different senses: (*a*) in the sense of Lwoff[13] to denote the induction of the lytic cycle of phage development, e.g., by ultraviolet light, and (*b*) in the sense of Monod and Cohn[14] to denote enzyme induction, e.g., in response to a substrate. Since each of these phenomena may represent release from repression exerted by a cytoplasmic agent,[15, 16] the use of identical terms may have some merit.

MATERIALS AND METHODS

BIOLOGICAL

Parental Strains of Bacteria.—The strains of *E. coli* employed here are listed in Table 1. Two strains incapable of fermenting galactose, and therefore known

TABLE 1

CHARACTERISTICS AND DERIVATION OF *Escherichia coli* STRAINS EMPLOYED

Strain	Derivation: Parent	Derivation: Process	Galactose phenotype: *K*	*T*	*E*	Original source	Donor
Sensitives							
C600			+	i^+	+	Ad[8]	Kr
W3110′	W3104	Selection	+	i^+	+	A	
W3104	W3104(λ)	UV	+	−	+	A	
Lysogens							
C600(λ)	C600	Infection with the indicated phage	+	i^+	+		Kr
C600(λ*imm*[434])	C600	Infection with the indicated phage	+	i^+	+		Lu
C600(434)	C600	Infection with the indicated phage	+	i^+	+		P
C600(82)	C600	Infection with the indicated phage	+	i^+	+		P
C600(21)	C600	Infection with the indicated phage	+	i^+	+		P
W3110′(λ)	W3110	Infection with the indicated phage	+	i^+	i^+	A	
W3092(λ)	W3100(λ)	UV	−	i^-	+	L	Ki
W3092(λ*imm*[434])	W3092(λ)	Infection	−	i^-	+	J	J
W3092A(λ)	W3092(λ)	Spontaneous	−	i^+	+	A	
W3104(λ)	W3110(λgal_4 +λ)	Segregation	i^+	−	+	L	Kr
Syngenotes							
W3104(λW3092+λ)	See text		+	i^+	+	A	
W3092(λW3104+λ)	See text		+	i^+	+	J	J
W3092(λW3092+λ+λ*imm*[434])	See text		−	i^-	+	A	

All bacterial strains are derivatives of *Escherichia coli* K12. The phage harbored by lysogens are indicated within parentheses and are described under *Materials and Methods*. The bacterial strain from which the transducing phage of each syngenote originally derived is indicated following the letter λ.

The phenotypes with respect to three enzymes under the genetic control of the galactose region, namely, galactokinase, galactose-1-phosphate uridyl transferase, and uridine diphosphogalactose 4-epimerase, are given below the letters K, T, and E, respectively. The presence (absence) of an enzyme in galactose-adapted cells, as determined by direct measurement of enzyme activity in extracts or by inference from enzymatic capacities of the parental strain, is indicated by a plus (minus) sign. Enzyme inducibility upon growth of the strain in the presence of 10^{-2} *M* D-galactose is indicated only where it has been directly determined, either by ourselves or by Kalckar, Kurahashi, and Jordan;[17] i^+ and i^- designate the inducible and constitutive state respectively, and the reader is referred to Table 2 for the quantitative significance of these terms.

Investigators' names have been abbreviated as follows: A, present authors; Ad, R. K. Appleyard; C, A. M. Campbell; J, E. Jordan; Ki, K. Kurahashi; Kr, A. D. Kaiser; Lu, S. E. Luria; L, E. M. Lederberg; P, K. J. Paigen.

as gal-negatives, are included: W3092, which lacks the capacity to synthesize galactokinase[18] owing to the gal_2 mutation[6] within the cistron designated "A"

by E. M. Lederberg,[19] and W3104, which lacks the capacity to synthesize galactose-1-phosphate uridyl transferase[17] owing to the gal_4^- mutation[6] within the "B" cistron.[19] The strains are not otherwise isogenic. Strain W3092 was derived by a single UV irradiation of the gal-positive strain W3100.[17] It differs from the wild type parent, not only in being incapable of galactokinase synthesis,[18] but also in that the transferase, inducible in W3100, is constitutive in W3092.[17] Spontaneous revertants of W3092(λ) to galactose-fermenting capacity are again inducible for transferase.[20] The above observations suggest an obligatory connection between the absence of galactokinase and constitutivity of transferase. Yet the connection is not obligatory, since a variant of W3092(λ), designated W3092A(λ), while still gal-negative, was found to be inducible for transferase. The nature of the differences among strains W3100(λ), W3092(λ), and W3092A(λ) is currently being investigated in this laboratory by E. Jordan. Strain W3104 was derived from a lysogen by a dose of UV lethal to the phage. The parent lysogen was a gal-negative segregant of a heterogenote consisting of W3110, a gal-positive strain, carrying lambda transducing the gal_4^- marker.[3] The gal-negative strains received were lysogenic for lambda and were freed of this phage either by UV treatment[21] or by displacement with the related phage λimm^{434}.[22] Sensitive clones were scored on basal EMB agar by the method of Lederberg and Lederberg.[23] A spontaneous gal-positive revertant of W3104 is designated here as W3110′ by reference to its parental wild type, W3110. The reversion is presumed to be due to back-mutation at the gal_4 locus rather than a suppressor mutation outside the galactose region because lambda-mediated transduction of genetic material from the supposed W3110′ into W3104 yields a gal-positive heterogenote.

The lambda prophages present in the lysogens may be characterized by the immunity they confer: strains C600(λ), W3092(λ), W3092A(λ), and W3104(λ) are each immune to phage obtained by induction from any of the other three. They are also immune to λ_{C71}.[24] They are all sensitive to λimm^{434}, described below, and to λ_{V2}, an inducing virulent mutant of lambda.[24] Phages λ_{C71} and λ_{V2} were supplied by Dr. Armin D. Kaiser. The lambda phage used for infection of strain W3110′ was obtained from a lysate of W3092(λ).

Phages *82* and *434*, described by Jacob and Wollman,[25] are related to lambda. Their prophage, like lambda prophage,[22, 26] locate close to the galactose region of the lysogenized bacterium.[54] A hybrid between lambda and *434*, obtained by Kaiser and Jacob,[7] is constructed so as to be isogenic with lambda except for the c_I region controlling prophage attachment site and immunity. This hybrid, designated λimm^{434}, is also capable of transducing the galactose region.[9] Phage *21*, while immunologically and genetically similar to lambda, is localized as prophage at a considerable distance from the galactose region.[25]

Preparation of Syngenotes.—Treatment of one suitable gal-negative bacterial strain with an LFT lambda lysate derived from another gal-negative strain with a complementary deficiency yields unstable clones of gal-positive heterogenotes recognizable on indicator agar. From these may be produced HFT lysates. An HFT lysate applied at high multiplicity to a lambda-sensitive derivative of the strain which served as source of the LFT lysate yields among the infected cells a high proportion of lysogenic homogenotes, recognized as such by their capacity to yield HFT lysates upon induction. Spotting of transducing lambda upon the

appropriate indicator bacteria insures that the intended marker is carried by the phage.

The syngenotes listed in Table 1 were prepared according to the steps indicated above as presented in detail by Morse, Lederberg, and Lederberg[6] and by Arber.[3]

For the rapid detection of gal-positive colonies against a crowded background of gal-negative colonies, tryptone-TTC-galactose agar of Arber[3] was used. The use of TTC-agar depends upon the ability of bacteria fermenting the added sugar to reduce the colorless 2,3,5-triphenyltetrazolium chloride (TTC) to the insoluble red dye formazan.[27] This agar sometimes gives misleading results, e.g., in the case of the various C600 lysogens, for reasons which remain to be explored. These strains, while galactose-negative on the TTC-agar, are galactose-positive on EMB-galactose agar.[1] TTC was obtained from the Dajac Laboratories Division of the Borden Co.

Syngenotes W3092(λW3104 + λ) and W3092(λW3092 + λ + λ*imm*^{434}) were prepared by transduction of W3092(λ*imm*^{434}) with the incidental elimination of the hybrid phage in the first case. Transducing phage from an HFT lysate was required for production of W3092(λW3092 + λ + λ*imm*^{434}). The source of the HFT lysate was the heterogenote W3104(λW3092 + λ).

Transducing phage was assayed on plates of tryptone-TTC-galactose agar, as described by Arber.[3] Helper phage derived from W3104(λ) and from W3092(λ) was used for assay of transducing phage with the recipient strains W3104 and W3092(λ*imm*^{434}) respectively. Lysates of each syngenote were demonstrated to be HFT with respect to the transduced markers they are reported to harbor. Lysates of each heterogenote were shown to be LFT with respect to the particular galactose markers characteristic of the bacterial host alone.

UV Irradiation en Masse for Phage Induction.[28]—Cells in the logarithmic phase of growth on tryptone-saline broth containing 0.01*M* $MgSO_4$[29] were harvested by centrifugation at 2000 × *g* for 10 minutes and resuspended in the UV-transparent irradiation medium of Campbell.[4] From the absorbance and volume of the resuspended culture, the irradiation period was calculated as indicated below. Cells were irradiated in a darkened room in a cylindrical vessel with vigorous mechanical stirring. They were then transferred to an equal volume of double-strength tryptone-saline-$MgSO_4$ broth in an Erlenmeyer flask of "low-actinic" glass (to prevent photoreactivation) and returned to the rotary shaker for incubation until cellular lysis occurred. An attempt was made to perform all operations, including centrifugation, at the same temperature, routinely 30°. At this temperature, lysis occurs at about 140 minutes after irradiation rather than at the 60 to 70 minutes characteristic of cells grown at the customary 37°.

Irradiation was from a "Mineralight" Senior Model ultraviolet lamp, claimed by the manufacturer to emit 110 mWatts/ft^2 at 2537 Å at a distance of 2 ft. from the light source. The lamp was allowed to warm up 15 minutes before use. Solutions to be irradiated were placed at a distance of 28 cm for the lamp where they may be expected to have received 56 ergs/mm^2/sec at 253.7 mμ. Irradiation time was calculated from the formula

$$t = 38\, A \cdot V \cdot r^{-2}\, (1 - e^{-4.8\, A \cdot V \cdot r^{-2}})^{-1}$$

where t = irradiation time in seconds at the above dose rate, A = absorbance of

the cell suspension at 650 mμ (1 cm light path) as measured in a model DU Beckman spectrophotometer, V = volume in ml of the cell suspension, and r = radius in cm of the cylindrical vessel. The factor in parentheses reduces to unity if the solution is essentially opaque to the incident UV. The formula represents an empirical result at infinite dilution corrected for finite absorbance by the Beer-Lambert Law. As the formula was generally applied under a limited range of conditions, namely, $A = 0.6$ (i.e., $5 \cdot 10^8$ viable cells/ml), V = 50–100 ml, and r = 6 cm, no corrections were made for variations in light-scattering.

Conventional Techniques of Virology.—Viable counts, infective centers, and active phage were determined on pour-plates as described by Adams.[30] By infective centers are meant those cells which give rise to one or more active phage particles as measured by plaques on the sensitive indicator strain W3104.

With the K-12 strains of *E. coli*, grown in tryptone broth at either 30° or 37°, 10^8 viable cells per ml correspond to an absorbance at 650 mμ of about 0.09 in the 1 cm light path of the model DU Beckman spectrophotometer. This absorbance value is three times the figure reported by Roberts *et al.*[31] The discrepancy may be due, at least partially, to differences in the growth medium used.

The efficiency of mass-induction as measured by the ratio of infective centers, following irradiation, to viable cells, prior to irradiation, was found to be generally less than 50 per cent, although irradiation caused immediate reduction in the viable count by more than 95 per cent. Post-irradiation lysogenic cells do not divide, but do continue to grow linearly, as seen from the linear increase in absorbance of the irradiated cultures (see figures). Cellular lysis, as measured by decrease in absorbance at 650 mμ, appeared eventually to destroy the majority of the irradiated cells.

In general, the W3104 indicator cells used for the assay of phage and infective centers were starved for about 1 hour in 0.01 M $MgSO_4$ before use.[29] To insure that phage samples taken during the period of cell lysis are not rendered inhomogeneous by the presence of strands of deoxyribonucleic acid, crystalline deoxyribonuclease (Worthington Co.) was routinely added to the cultures at about the 100th minute after irradiation at a final concentration of 1 μg protein per ml. Phage were diluted in a lambda-diluent described by Kaiser[32] prior to assay. Intracellular phage were assayed in premature lysates obtained by vigorously shaking culture samples with a few drops of chloroform.[33]

BIOCHEMICAL

Enzyme assays.— (*a*) *Galactose-1-phosphate uridyl transferase:* The assays of this enzyme, hereafter abbreviated transferase, and of uridine diphosphogalactose 4-epimerase, hereafter abbreviated epimerase, are as described by Kalckar, Kurahashi, and Jordan.[17] The methods ultimately depend on the spectrophotometric measurement of pyridine nucleotide reduction in coupled systems. Both enzymes catalyze the interconversion of uridine diphosphoglucose and uridine diphosphogalactose; epimerase does this directly, transferase by an exchange of phosphorylated sugars. The specific measurement of transferase activity is accomplished by assaying the formation of α-D-glucose-1-phosphate dependent upon the presence of uridine diphosphoglucose and α-D-galactose-1-phosphate. In the presence of these two substrates and excess phosphoglucomutase, glucose-1,6-diphosphate,

glucose-6-phosphate dehydrogenase, and TPN, the initial linear rate of TPN reduction is a measure of transferase activity. It is occasionally necessary to subtract a low rate of TPN reduction observed prior to the addition of the last substrate (preferably galactose-1-phosphate). Routinely, proportionality to bacterial extract concentration was demonstrated. Reactions were initially run in the compartment of a model DU Beckman spectrophotometer without temperature control. In later experiments, incubation was performed in the water-jacketed compartment of a model-14 Cary recording spectrophotometer, in which the cuvettes were maintained at approximately 30°.

The purified enzymes used in the assay system were shown to catalyze the reduction of TPN in mole for mole stoichiometry with the amount of glucose-1-phosphate added. Once formed, the TPNH is not reoxidized at an appreciable rate even in the presence of crude bacterial extracts. The presence of 6-phosphogluconic acid dehydrogenase in these extracts does permit reduction of a second mole of TPN for every mole originally reduced. In general, this contaminating enzyme does not affect the initial linear rate of TPNH formation, as was shown by the failure of an extract of the transferaseless strain W3104(λ) to increase the apparent activity of a sample of transferase purified free of 6-phosphogluconic acid dehydrogenase activity. The sample of purified transferase was the gift of Dr. Kiyoshi Kurahashi.

The assay enzyme, phosphoglucomutase, was either the crystalline enzyme obtained from rabbit skeletal muscle and generously furnished by Dr. Victor Najjar or an amorphous preparation purified according to the method of Najjar[34] through the penultimate step. Each cuvette received 1 μg of this protein in a final volume of 0.6 ml along with $6 \cdot 10^{-4}$ μmoles of glucose-1,6-diphosphate. This is sufficient to insure a rate of TPN reduction by glucose-1-phosphate in the transferase assay system corresponding to an absorbance change at 340 mμ of 0.200 per min.

Glucose-6-phosphate dehydrogenase was Type III (substantially free of hexokinase and 6-phosphogluconic acid dehydrogenase) purchased from the Sigma Chemical Company. Each cuvette received 0.05 Kornberg units.[35] Both of the purified enzymes employed in the assay were made up separately in 10 mg per ml crystalline bovine plasma albumin (Armour Company) and stored at −60°.

Crystalline α-D-galactose-1-phosphate, dipotassium salt, substantially free of glucose-1-phosphate; crystalline glucose-1-phosphate, dipotassium salt; uridine diphosphoglucose, sodium salt; and TPN, sodium salt were purchased from the Sigma Chemical Company. The barium salt of glucose-1,6-diphosphate was generously donated by Dr. Luis Leloir of the Instituto de Investigaciones Bioquímicas, Buenos Aires through Dr. Victor Ginsberg of the National Institutes of Health, Bethesda, Maryland. It was converted to the sodium salt before use and assayed with phosphoglucomutase by reference to its Michaelis constant of $5 \cdot 10^{-7}$ *M*.[34] A second sample of partially purified glucose-1,6-diphosphate was obtained through the kindness of Dr. Herbert C. Friedmann, who prepared it through the first barium fractionation step in the method of Leloir *et al.*[36]

(*b*) *Uridine diphosphogalactose 4-epimerase:* This enzyme was assayed by the two-step procedure described by Kalckar *et al.*[17] Incubation mixtures preincubated at 30° with added uridine diphosphogalactose were heated at 100° for 90 seconds and then assayed for uridine diphosphoglucose by spectrophotometric

measurement of the DPN reduced upon the addition of DPN and uridine diphosphoglucose dehydrogenase. The dehydrogenase was purchased from the Sigma Chemical Company as Type III. Synthetic uridine diphosphogalactose, lithium salt, was the gift of Dr. Herman M. Kalckar. A sample was also prepared enzymatically[37] by Dr. Elizabeth Maxwell in collaboration with one of us. DPN was purchased from the Sigma Chemical Company.

(*c*) *Uridine diphosphoglucose pyrophosphorylase:* This enzyme, like galactose-1-phosphate uridyl transferase, is also a uridyl transferase, the acceptor molecule here being pyrophosphate. The assays of the two enzymes, dependent upon measurement of glucose-1-phosphate formed, are similar.[38] It was found essential to use a high concentration of pyrophosphate ($6 \cdot 10^{-3}$ *M*) in order to obtain a linear time course of reaction. Pyrophosphate is apparently rapidly hydrolyzed in crude *E. coli* extracts.

(*d*) *Beta-galactosidase:* The method of Lederberg, employing *o*-nitrophenyl-β-D-galactoside, *o*NPG, as chromogenic substrate was used.[39] Incubations were carried out at 37°. Calculations of enzyme activity are based on an extinction coefficient at 420 mμ of $\epsilon = 4.6 \cdot 10^3$ cm^2 mmole^{-1} for *o*-nitrophenol in 0.67 *M* Na_2CO_3. *o*NPG was purchased from the California Corporation for Biochemical Research and *o*-nitrophenol was from Eastman Organic Chemicals.

(*e*) *6-Phosphogluconic acid dehydrogenase:* This enzyme was assayed by the spectrophotometric procedure of Horecker and Smyrniotis,[40] incubations being performed at approximately 30° in the compartment of a model-14 Cary recording spectrophotometer. The sodium salt of 6-phosphogluconic acid was purchased from the Sigma Chemical Company.

Conditions of Enzyme Extraction.—The following procedure has been used throughout the experimental work. Culture samples ($3–5 \cdot 10^8$ viable cells per ml) of approximately 20 ml are centrifuged for two minutes in 12 ml centrifuge tubes in a desk-top centrifuge to sediment the cells. At this point, the drained pellets may be frozen at −60°. The pellets are resuspended in $^1/_2$ to 1 ml of the potassium phosphate-$MgSO_4$ buffer identical to the medium in which cells are irradiated.[4] One-half ml aliquots of the suspension are transferred to lusteroid tubes in an ice bath and each is submitted to 45 seconds of sonication with the 6 mm diameter probe of the Mullard 20 kc per sec sonicator. The sonic extracts were centrifuged in stainless steel tubes for one hour at 144,000 × *g* in the refrigerated Spinco preparative ultracentrifuge. The supernatant fluid is then assayed or stored at −60° to be assayed at convenience.

Sonication is the method of cell disintegration used by Kurahashi and Sugimura for purification of the enzyme[41] and is superior or equivalent to alternative methods tried.[42] In exploratory studies of enzyme stability and extractability in small samples, attention was largely confined to transferase activity released by sonication. Below are briefly summarized the results of these studies. The bacterial strains used were either W3092(λ) or W3104(λW3092 + λ) grown in the absence of galactose.

Transferase appears to be a soluble enzyme. All of the transferase activity present in a crude bacterial extract, prepared through sonication as described above, remains in the supernatant fluid after centrifugation at 144,000 × *g* for one hour. Increasing the sonication time to 2 minutes does not appreciably alter the amount

of enzyme activity or protein extracted. Further sonication causes some enzyme inactivation. Sonication for 30 seconds appears adequate to cause maximal extraction of enzyme activity from cells which have not been irradiated. Cells which have been induced by irradiation are fragile just prior to lysis and require less sonication, but the transferase they release exhibits no increased lability to further sonication. The transferase in extracts prepared by harvesting induced lysogens towards the end of the latent period and allowing them to lyse spontaneously in a small volume of buffer is of no greater specific activity than is attained by sonication of the same cells. The presence of 10 mg per ml bovine plasma albumin or 10^{-2} *M* galactose during sonication is without effect.

An extract subjected to 5 cycles of freezing and thawing suffered no detectable loss of transferase activity. Packed cells and enzyme extracts have been successfully stored at $-60°$ for several days without losing enzymatic activity.

Reproducibility of enzyme extraction and assay was determined in an early experiment by assaying five separately sonicated aliquots of a bacterial culture. The mean specific transferase activity and standard deviation were 4.3 ± 1.2 μM TPNH/hr/mg protein.

Centrifugation of the sonicates serves to clarify them and is assumed to remove a constant proportion of the total protein which is then neglected in calculations of specific activity. This assumption is only approximately correct. Protein removed by centrifugation represents between 20 and 40 per cent of the total.

Protein Determination.—Sonicates containing 1 to 2 mg of bacterial protein per ml were freed of contaminating tryptone by precipitation at 0° with 0.35 *M* trichloroacetic acid. The protein of the tryptone broth is precipitated with less than three per cent the efficiency of precipitation of bacterial protein. The protein pellets were washed once with 2 ml 0.35 *M* trichloroacetic acid by centrifugation and then dissolved in dilute alkali and assayed for protein by a simple microbiuret procedure.[43]

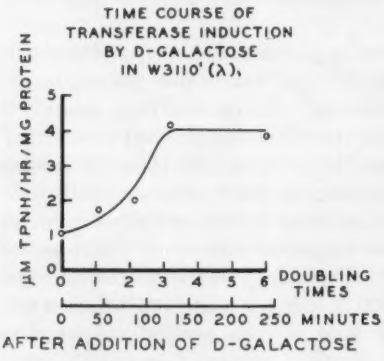


FIG. 1.—To a logarithmically growing culture of W3110′(λ) in tryptone broth $+10^{-2}$ *M* $MgSO_4$, at 30°, D-galactose was added at the start of the experiment to a final concentration of 10^{-2} *M*. The culture was maintained in logarithmic growth by dilution in a fresh medium at the time of each sampling.

Enzyme Induction by D-*Galactose and* D-*Fucose.*—It can be seen from Figure 1 that by the third generation of logarithmic growth in the presence of 10^{-2} *M* D-galactose the transferase activity of strain W3110′ is maximally induced. The ratio of transferase specific activities, maximally induced to uninduced, is low, 4.0 in this case; in the strains tested, this induction ratio does not exceed a value of 10 whether D-galactose or D-fucose (6-deoxygalactose) is used as the inducing sugar (Table 2). A strain in which the induction ratio reaches or exceeds a value of 2 will be considered inducible and will be designated i^+. Where the ratio does not reach this value, and if transferase activity is appreciable, the strain will be considered constitutive and will

TABLE 2

TRANSFERASE INDUCIBILITY

Part	Strain	Inducing sugar: None	Inducing sugar: D-galactose	Inducing sugar: D-fucose	Maximum inducibility ratio
A	W3110′	0.66 1.00*	4.4	...	6.7
A,B	W3110′(λ)	1.0 0.92*	4.0	2.9	4.0
B	W3104	<0.02	<0.02	...	...
B	W3092(λ)	2.8 2.3	2.9 2.3	2.7	1.0
	W3092A(λ)	1.4	11.1	...	7.9
B	W3104(λW3092+λ)	1.3 2.6	5.0 12.1	7.2	5.5
B	W3092(λW3104+λ)	1.0	8.3	6.2	8.3
	W3092(λW3092+λ+λ*imm*^{434})	5.1 8.3	7.8 9.2	8.1	1.6
	C600	1.3	5.4	...	4.2
	C600(λ)	1.1	5.1	...	4.6

Cells were grown at 30° in tryptone-saline-$MgSO_4$ broth[29] and harvested in the logarithmic phase after more than four generations in the presence of 10^{-2} *M* inducer. The values of the first three columns are specific activities of transferase in μM TPNH/hr/mg protein. Values marked by an asterisk are the average of four separate determinations. The last column expresses the ratio of maximally induced activity to uninduced activity. These ratios are more significant than comparisons of specific activity values in different rows.

be designated i^-.

The conditions of transferase induction by D-galactose appear inadequate to induce β-galactosidase much above its basal level in those strains in which this enzyme was assayed. The enzyme may be induced to much higher levels by an inducer such as thiomethyl-β-D-galactoside.

D-galactose was purchased from Pfanstiehl Laboratories and D-fucose from the K and K Laboratories, Jamaica 33, New York.

RESULTS

Transduction and Dominance of Transferase Inducibility.—Small differences in the specific activity of transferase form the basis of the distinction between i^+ and i^- strains (Table 2), whether induction is effected by D-galactose or by D-fucose

TABLE 3

EFFECT ON TRANSFERASE ACTIVITY OF MIXING BACTERIAL EXTRACTS

No.	Source of extract	Transferase specific activity: Found	Transferase specific activity: Calculated
1	(*a*) Uninduced cells	0.53	...
	(*b*) D-galactose-induced cells	4.0	...
	(*c*) Mixture of (*a*) and (*b*) extracts (3:1 by protein)	0.92	0.80
2	(*a*) Unirradiated cells	4.2	...
	(*b*) Irradiated cells at start of lysis	11.6	...
	(*c*) Incubated mixture of (*a*) and (*b*) extracts (3:2 by protein)	7.0	7.1

In experiment No. 1, designed to determine whether extracts of uninduced cells inhibit the assay of an extract of induced cells, the bacteria were W3110′(λ) and were induced by growth in the presence of 10^{-2} *M* D-galactose. The mixture was not preincubated. Specific activities are expressed in μM TPNH/hr/mg protein.

In experiment No. 2, designed to determine whether the lytic enzyme of a UV-induced cell extract can increase the transferase activity of an unirradiated cell extract, the bacteria used were W3092(λW3092+λ+λ*imm*^{434}). Crude uncentrifuged extracts were preincubated, either separately or combined, for 30 min at 30°, then assayed without centrifugation. Negligible loss in enzyme activity occurs during preincubation. Specific activities are expressed in μM TPNH/hr/mg total protein.

(6-deoxygalactose). We can ascertain, by a mixing experiment such as shown in Table 3, experiment 1, that the specific activity differences between an i^+ and an i^- strain are *not* due to the presence of an inhibitor of enzyme assay in extracts of the i^+ strain which is absent or lower in concentration in extracts of the i^- strain. The experiment shows that the activity of mixed extracts is simply additive.

In two further respects, the inducibility of transferase behaves like other instances of enzyme inducibility which have been more intensively studied. Firstly, a gene determining inducibility of transferase, a "gene of regulation," appears to be distinct from a "gene of structure" specifying the enzyme.[44] Thus, from Part B of Table 2, it can be seen that the transferaseless strain W3104, derived from the i^+ strain W3110, appears to retain a functional inducibility gene since the heterogenote W3092(λW3104 + λ) is i^+. This observation suggests the existence of a transducible inducibility locus. The restrictive nature of lambda transductions, noted earlier, places this new i-gene within the *gal* region; just as in the case of the *lac* region the corresponding i-gene is found within that region.[44] A second point apparent from the enzyme levels of the two reciprocal heterogenotes is that irrespective of how the i^+ and i^- factors are distributed between host and transducing phage, the heterogenote is always inducible, i.e., the i^+ state is dominant over the i^- counterpart. We refrain from speaking of alleles where allelism remains to be established. Dominance of the i^+ state is consistent with the theory, first proposed by Vogel,[46] that inducibility results from the existence of a specific repressor of enzyme synthesis which is antagonized by inducers, while complete constitutivity results from the absence of repressor.

Absence of Lysogenic Conversion by Normal Lambda of Transferase Inducibility.—The proximity of the attachment site of lambda prophage to the *gal* region suggests the possibility of an influence of lambda prophage on the expression of genes within that region. Specifically, the possibility that the differential rate of transferase synthesis might be altered by lysogenization with lambda was examined in the progeny of four separately lysogenized cells of W3110′. The averages reported in Part A of Table 2 reveal no significant effect of lysogenization with lambda upon transferase levels in these inducible cells.

Increased Transferase Activity following Induction of Transducing Lambda.—Two reciprocal heterogenotes and a homogenote have been prepared in this laboratory, the heterogenotes being inducible, the homogenote constitutive or nearly so. The specific activity of transferase plotted against time after the initiation of phage development by UV irradiation is shown, for each of these strains, in Figure 2. These experiments, carried out at 30°, yield, upon compression of the time scale, kinetic curves identical in shape to those obtained when the experiments are carried out at 37°, but the extended latent period at 30° is somewhat more convenient for repeated samplings. For this reason, all the experiments presented here were run at the lower temperature.

Each graph is provided with a caption indicating the presumptive genotype of the strain; allelism of alternative inducibility states is assumed merely for simplicity of presentation.

Preliminary attempts at quantitative measurement of transducing lambda have not proved sufficiently accurate to be reported here.

The absorbance measurements reported were made immediately upon sampling

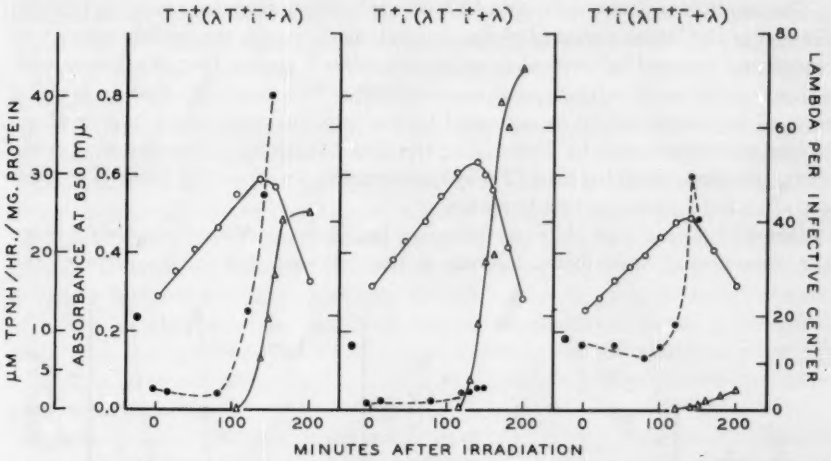


FIG. 2.—Transferase activity following induction of phage replication in syngenotes. The bacterial strains employed are, from left to right, W3104(λW3092 + λ), W3092(λW3104 + λ), and W3092(λW3092 + λ + λ*imm*^{434}). The key to the symbols used is provided in Figure 3. The ratios of infective centers measured promptly following irradiation to viable count prior to irradiation for the three experiments are, from left to right, 0.39, 0.39, and 0.45. Lambda titrations were performed with the indicator strain W3104 and therefore in the case of the homogenote include lambda and λ*imm*^{434}. The low phage yield with the homogenote is not atypical for this strain.

The unconnected symbols representing transferase specific activity drawn to the left of zero minutes give the values obtained for parallel cultures grown in the presence of 10^{-2} *M* D-galactose for at least 6 doubling times.

the culture; after the initiation of lysis, cells allowed to stand in the cuvette lyse more rapidly than in the incubating culture itself.

In each case, the specific activity of transferase increases during the final third of the latent period of phage development. This increase is most striking in the case of the heterogenote in which the T^+i^- transducing phage replicates against the background of a T^-i^+ host. The reciprocal heterogenote shows only a small, but nevertheless significant, increase in enzyme activity. If transducing genetic material were functional during the latent period of the phage development, in the sense of initiating enzyme synthesis, we might expect the rate of transferase synthesis to increase in the first-mentioned heterogenote, but no such rise should occur in the reciprocal heterogenote. In the latter, we might expect an increased repression of enzyme synthesis owing to replication of the i^+ gene. It would appear that an explanation other than increased gene dosage must be invoked to explain the fact that transferase activity increases in both heterogenotes upon phage induction.

Experiments have been performed in which the transferase of the heterogenote W3104(λW3092 + λ) was induced prior to irradiation by growth in the presence of 10^{-2} *M* D-galactose. During the latent period of phage development, which was also allowed to proceed in the presence of the sugar, the specific activity of transferase increased above the normal galactose-induced level. The time course of this enzyme increase resembles that seen following induction of the homogenote of Figure 2.

The rise in transferase activity which occurs in the homogenote is again towards the end of the latent period of phage development. Again the specific activity of transferase attained subsequent to phage induction is greater than is attained with either D-galactose or D-fucose as enzyme inducer. The possibility that an inactive form of transferase might be activated by the lytic enzyme present in cells about to lyse was investigated by determining the specific activity of transferase in crude extracts preincubated together (Table 3, experiment No. 2). Additivity of enzyme activities fail to support this hypothesis.

Increased Transferase Activity following Induction of Normal Lambda.—Since the induction of transducing lambda in the previous experiments involves the

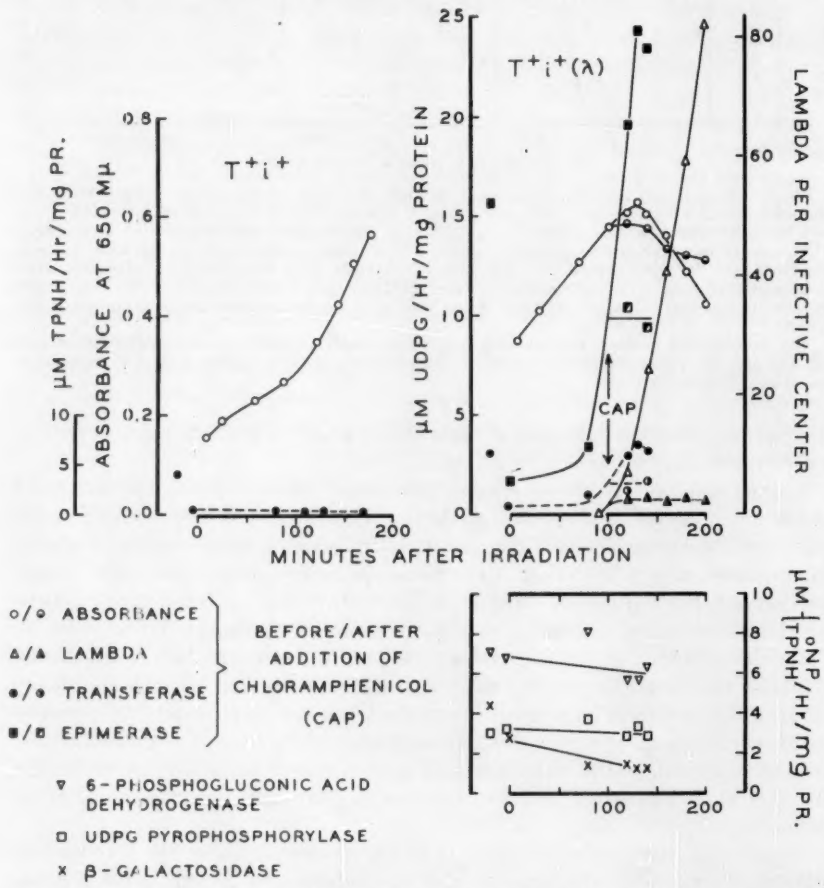


FIG. 3.—Enzyme activities following irradiation of lambda-sensitive and lambda-lysogenic *E. coli*. The bacterial strains employed are W3110′ (left) and W3110′ (λ) (right). The ratio of infective centers, measured promptly following irradiation of the lysogen, to viable count measured prior to irradiation is 0.42. The scales are the same as in Figure 2. For transferase, UDPG pyrophosphorylase, and 6-phosphogluconic acid dehydrogenase activity, refer to the scales labeled μM TPNH/hr/mg protein; for epimerase activity, μM UDPG/hr/mg protein; for beta-galactosidase activity, μM oNP/hr/mg protein. As in Figure 2, the unconnected symbols representing enzyme specific activities drawn to the left of zero minutes give the values obtained for parallel cultures grown in the presence of 10^{-2} M D-galactose for about 6 doubling times. At 100 minutes after irradiation of the lysogen, a portion of the culture was transferred to a separate containing flask chloramphenicol (Parke, Davis and Co.) at a final concentration of 40 μg/ml.

simultaneous induction of helper phage, it is necessary to determine the effect upon transferase activity of induction of normal lambda as well as the effect of the inducing irradiation alone upon a lambda-sensitive strain. Irradiation of a sensitive strain, either W3110′ (Fig. 3, left) or C600 leads to a lag period after which the culture resumes logarithmic growth. Irradiation does not serve to bring about an increased activity of transferase.

Irradiation of an active lysogen, W3110′(λ) or C600(λ), does bring about a rise in the specific activity of transferase. This is shown by the dashed curve on the right in Figure 3. The line is drawn partly on the basis of two replicate experiments. In these experiments, transferase specific activity increased, by the time of lysis, 8 fold and 12 fold, respectively, as compared with 9 fold in the experiment of Figure 3. Here, as in the case of the induced syngenotes of Figure 2, the rise in transferase activity occurs late. Also upon irradiation of the constitutive lysogen W3092(λ), increases of two to three fold in the specific activity of transferase have been found to occur.

Since induction of normal lambda alone in i^+ or i^- normal lysogens is capable of increasing transferase activity, this raises the question of how much of what occurs upon induction of a lysogenic syngenote is due to induction of the helper phage.

Limitation of Specific Activity Increases, following Induction of Normal Lambda, to Enzymes of the Gal Region; Inhibition by Chloramphenicol.—A possible limitation of the enzymes whose activities are increased following lambda induction to those genetically determined by the *gal* region is suggested, in part, by the observation of Buttin *et al.*[5] that galactokinase activity is increased by lambda induction just as is transferase activity. The hypothesis is supported by a study of the activities of enzymes under separate genetic control and one additional enzyme determined by the *gal* region, uridine diphosphogalactose 4-epimerase. The enzymes assayed in the experiment of Figure 3 include, apart from transferase and epimerase, two enzymes involved in galactose or galactoside metabolism (uridine diphosphoglucose pyrophosphorylase and β-galactosidase). The last mentioned, like transferase and epimerase in this strain, is inducible, although D-galactose under the conditions of the experiment is an exceedingly poor inducer. A third enzyme, 6-phosphogluconic acid dehydrogenase, is of particular interest since its activity may affect the measurement of transferase activity, of which more will be said later. Most striking about the curves of Figure 3 is the contrast between enzymes determined by genes lying within the *gal* region and those determined by genes outside the *gal* region. Following lambda induction, the specific activities of the former enzymes increase together (upper graph), whereas those of the latter group decrease slightly (lower graph).

Epimerase activity is considerably greater than transferase activity and may even be underestimated in the experiment of Figure 3. Linearity of the assay with time of incubation of substrate with sonicate was not attained, although the two-step assay of Kalckar *et al.*[17] was used. The shape of the epimerase specific activity curve is probably not seriously affected by the inaccuracy thus introduced.

The reliability of transferase assays may be criticized on the basis of two considerations. The first of these is that the number of molecules of TPN reduced by each molecule of glucose-1-phosphate depends upon the contribution of 6-phosphogluconic acid dehydrogenase to the oxidation of the sugar phosphate. This con-

tribution is not necessarily constant and unless taken into account may lead to an error by a factor of as much as two. A slight decline in 6-phosphogluconic acid dehydrogenase specific activity following irradiation has been found to occur not only in the lysogen of Figure 3, but also in the sensitive strains and in the heterogenotes. Therefore, the error introduced by the changing proportion of 6-phosphogluconic acid dehydrogenase activity can only lead to an underestimate of the transferase specific activity increases reported here.

A second and more serious concern is the specificity of the assay designed to measure transferase. It is possible to write two reactions, catalyzed by one or perhaps two pyrophosphorylases, which sum to yield an over-all reaction identical to the one catalyzed by the galactose-1-phosphate uridyl transferase:

(1) UDPG + PP = UTP + G-1-P
(2) Gal-1-P + UTP = PP + UDPGal
Sum: UDPG + Gal-1-P = UDPGal + G-1-P

An enzymatic catalysis of reaction (1) has already been demonstrated by Kurahashi[18] to occur in extracts of *E. coli* K-12. Reaction (2) is known to occur in extracts of galactose-adapted yeast.[47] On the assumption that reaction (2) also occurs in *E. coli* extracts, an apparent change in the activity of galactose-1-phosphate uridyl transferase could be due to an alteration of pyrophosphorylase activity. The assays of uridine diphosphoglucose pyrophosphorylase of Figure 3, showing no specific activity increase subsequent to phage induction, diminish the possibility of such an artifact.

The increase in transferase or epimerase activity can be halted abruptly by addition of chloramphenicol to the culture (Fig. 3). Since protein synthesis is thereby specifically inhibited,[48] the observed increases in enzyme activity must represent, or at least be shortly preceded by, the synthesis of protein.

In the experiment of Figure 3, the time of addition of chloramphenicol is such as to largely dissociate the rise in enzyme activities from the formation of mature phage. A recent observation by Dr. Maurice Bessman that under a nitrogen atmosphere the formation of phage-induced deoxyguanylate kinase may occur in the absence of the synthesis of the majority of phage proteins[49] suggested to us the possibility of dissociating bacterial from viral protein synthesis in this way. However, in preliminary experiments, appreciable increases in the specific activity of transferase were not observed when the irradiated lysogen W3110′(λ) was maintained anaerobically.

Relation of Transferase Activity Increase to Prophage-Attachment Site following Induction of Lambda-Related Phages.—The transferase activity increase upon phage induction has been examined to determine the phage specificity of the phenomenon. The phages investigated are related to lambda as shown by certain similarities of phenotype and by their ability to undergo genetic recombination with lambda.[7, 25] They are all lysogenizing and become associated as prophage each with a specific locus along the bacterial chromosome. They are inducible by UV-irradiation. The locations of the prophages, determined by Jacob and Wollman,[54] are as follows: prophage *82* lies closest to *gal* region, next is lambda, then *434* (and λ*imm*^{434}) closely following; prophage *21* is located somewhat further along the chromosome.

Of the phages listed in Table 4, those which become situated in closest proximity to the *gal* region cause increased production of transferase following irradiation. On the other hand, induction of phage *21*, which lies at some distance from the *gal* region, fails to alter the level of transferase. The prophages prior to induction are without effect. While the results with phage *82* are ambiguous (owing perhaps

TABLE 4

TRANSFERASE ACTIVITY FOLLOWING INDUCTION OF LAMBDA AND RELATED PHAGES

Phage	Per cent viable cells induced	Transferase Specific Activity	
		Cells not irradiated	At time of lysis
None	..	1.3	...
82	21	1.3	1.7
λ	50	1.1	5.0
λ*imm*^{434}	41	1.4	4.1
434	38	1.2	3.0
21	69	1.1	1.1

The phages were initially present as prophage in strain C600. They are listed in order of increasing distance between the site of prophage localization and the gal region of the host. Infective centers, used in calculating per cent viable cells induced, were determined with the indicator bacteria C600 for *82*, λ*imm*^{434}, and *21*, C600(λ) for *434* and W3104 for λ. Transferase specific activity is in μM TPNH/hr/mg protein.

to the fact that the efficiency of induction is low), it would appear that alterations in the phage genome do not necessarily abolish the phenomenon of transferase increase, provided the prophage is not displaced too far from the *gal* region.

Construction of reciprocal hybrids between phages *21* and lambda (*21imm*$^{\lambda}$ and λ*imm*^{21}) should permit a more decisive evaluation of the role of prophage localization site in determining the capacity of a lysogenizing phage to cause increases in enzymes of the *gal* region after its induction.

DISCUSSION

The ontogeny of lambda during the lytic cycle is accomplished through the orderly functioning of phage genes whose capacities are latent in the prophage. It is of interest to know how a segment of bacterial genome behaves at various stages in the life of lambda when present as an insertion in the genome of the phage. Following the introduction of a transducing *gal*$^{+}$ determinant into a recipient cell, phenotypic expression has been found by several workers to occur almost immediately[5, 50, 51] and it persists following reduction to prophage. Even when the vegetative replication of phage has been initiated, the transduced genes apparently continue to function since they are capable of causing a further increase in enzyme synthesis, e.g., in the induced heterogenote W3104(λW3092 + λ) of Figure 2. Thus, throughout these stages in phage development, the transduced bacterial genes lying within the phage genome maintain their capacity to initiate enzyme synthesis.

The functioning of transduced genes is not, however, independent of the state of the phage. On the contrary, a series of experiments on galactokinase transduction into kinaseless recipient bacteria (lysogenic or sensitive to lambda) has led Buttin *et al.*[5] to conclude that when these bacteria are not or are no longer immune to lambda, transduced kinase synthesis, normally inducible, becomes constitutive.

In the course of our own studies and those of Buttin *et al.*, it was discovered that the phage genome can also affect the functioning of bacterial genes not inserted

within the phage genome but merely associated with it. The bacterial genes affected, those of the *gal* region, lie near the site of prophage localization and are influenced only upon phage induction (Fig. 3) and not by the mere presence of prophage (Table 2, part A, and Table 4). Because of the relative simplicity of the phenomenon, our primary concern has been a description of the influence which the induction of normal lambda and related phages exerts upon the *gal* region.

The increases in enzyme activities which follow induction of lambda are interpreted as reflecting the differential rate of synthesis of the proteins involved. The ability of chloramphenicol to halt the enzyme increases once they have started supports this interpretation. Results of mixing experiments (Table 3, Exp. No. 2) and the narrowness of phage specificity offer strong evidence against zymogen activation by a lytic enzyme or other agent formed in the course of phage development.

Prior to lysis of phage-infected *E. coli*., leakage of various substances from the cells is known to occur.[53] It might be argued that, during the latent period of phage development, loss of a repressor (or a substance required for repressor formation) could be responsible for the apparent derepression of enzymes of the *gal* region. This argument does not seem tenable in view of the ineffectiveness of phage *21*, closely related to lambda, in provoking the enzyme increases, although the phage matures and brings about lysis of the host.

The enzymes whose specific activities are increased following induction of lambda, viz., kinase, transferase, and epimerase, belong to one biochemical pathway and are controlled by a cluster of genes collectively called the *gal* region. The levels of all three enzymes are diminished[17] by single point mutations within a cistron of the *gal* region designated "C."[19] Group "C" mutations are noncomplementary with all mutations in cistrons "A," "B," or "C."[19] The proposal by Jacob *et al.*[45] that the genes of the *gal* region constitute an "operon," i.e., a genetic region under the coordinated pleiotropic control of a single "operator gene," would appear to be based on these properties of mutations in the "C" cistron. According to the concept of Jacob *et al.*[45] that the operator (or a cytoplasmic replica of it) is the seat at which repression is exerted, the enzymes of a sequence governed by the same operator should not be capable of separate induction. The kinaseless strain W3092 in which a previous report[17] and Table 2 show transferase to be constitutive and in which Kalckar *et al.*[17] have reported epimerase to be inducible may present a paradox and is therefore the subject of reinvestigation. The inducibility of at least transferase is affected by mutation within a presumed "gene of structure" determining kinase and has suggested an auxiliary role of kinase in controlling the formation of or sensitivity to a repressor substance.[52] Whether or not transferase inducibility is under separate control, the fact remains that all three enzymes of the *gal* region undergo a concerted increase following induction of lambda.

A survey of a few lambda-related lysogenizing phages reveals that the effect we are studying is sensitive to changes in prophage localization. This observation, but more particularly, some ingenious experiments reported by the group in Paris,[5] leads us to conclude that the host-phage interaction responsible for initiating increased enzyme activity is not mediated by a cytoplasmic agent. The experiments just mentioned compare kinase levels, which follow lambda induction, in partially diploid lysogens heterozygous at the *gal* region and in which the prophage is as-

sociated exclusively with either the *gal*$^+$ or the *gal*$_2^-$ bacterial chromosome. Synthesis of kinase becomes constitutive only where lambda prophage was originally associated with the *gal*$^+$ chromosomal segment.

The ability of lambda induction to cause coordinate increases in the same enzymes whose coordinate expression is presumed to be controlled by a cytoplasmic repressor and its receptor, the operator cistron "C," suggests that lambda induction affects either the repressor substance or the operator gene. As already indicated, available evidence appears to preclude the mediation of any cytoplasmic agent. We are therefore inclined to ascribe the enzyme increases, which occur upon lambda induction, to a direct interaction between a portion of the lambda genome and the "C" cistron of the bacterial *gal* region. Since lambda induction in i$^+$ lysogens has on occasion resulted in enzyme levels in excess of those achieved by induction with D-galactose or D-fucose, we must assume that the derepression normally afforded by these sugars is incomplete. The increases in transferase levels observed upon lambda induction in the constitutive strain W3092 require us to assume that transferase synthesis is to some extent repressed in this strain but not subject to derepression by either of the above sugars. Further experiments are required to determine the validity of the hypothesis that an operon of the host and a portion of the phage interact upon induction.

A number of factors may be expected to influence the specific activity of transferase following the induction of transducing lambda in lysogenic syngenotes. Not only may there be position effects due to surrounding genetic material of the transducing phage and perhaps the associated genetic material of helper phage, but there may also be dosage effects due to the increasing numbers of presumably functional genes determining enzyme structure and inducibility.

The large differences in the two reciprocal heterogenotes of Figure 2 between transferase levels following phage induction might be most simply attributed to the effects of gene dosage. Assessment of the role of gene dosage must, however, await less ambiguous experiments. The problem might be somewhat simplified if galactose markers could be inserted into the genome of a prophage which locates some distance from the *gal* region, e.g., λgal^+imm^{21}. Such a phage would be recognized by its capacity to transduce the *gal*$^+$ marker and confer immunity to phage *21* and not to lambda.

Lysogenizing phages which locate close to the genetic determinants regulating additional groups of enzymes and perhaps viruses inhabiting the nuclei of animal cells deserve attention to ascertain how general the phenomenon described here may be.

Summary.—(1) Evidence is presented for genetic regulation in *Escherichia coli* of the inducibility of galactose-1-phosphate uridyl transferase, inducibility being dominant over constitutivity. The determinant is transduced by lambda phage and is therefore presumed to be located within the *gal* region. (2) Where a gene for functional transferase synthesis is present in the transducing phage, induction of phage multiplication in cells doubly lysogenic for normal and transducing lambda is shown to cause a rise in transferase specific activity to levels higher than are attained by enzyme induction with D-galactose or D-fucose. Smaller enzyme increases occur where the transducing phage does not contribute a gene for the synthesis of enzymatically active transferase. (3) Evidence is offered, in part based

on the work of Buttin, Jacob, and Monod,[5] that induction of normal lambda specifically increases the differential rate of synthesis of the sequence of enzymes determined by the *gal* region of the host, viz. galactokinase, galactose-1-phosphate uridyl transferase, and uridine diphosphogalactose 4-epimerase. (4) Studies of lambda-related phages suggest that prophage localization site (near the *gal* region in the case of lambda[23]) is important in determining the effect of phage induction upon the subsequent activity of *gal* region enzymes. (5) The hypothesis is offered that an interaction between the "C" cistron of the bacterial *gal* region,[11] considered as the operator gene of the *gal* operon,[45] and a portion of the phage genome is responsible for the effects of phage induction upon *gal* region enzyme levels. Unresolved related problems are briefly discussed.

We are much indebted to those investigators whose names are listed in Table 1 (as donors) for providing us with bacteria and phage and for advice on matters of technique. Dr. Philip E. Hartman has also materially aided our understanding of virological aspects of this work.

We are again pleased to acknowledge our considerable debt to Dr. Herman M. Kalckar for advice and encouragement.

Our thanks are also due to Miss Elke Jordan, Mr. David M. Shapiro, and Dr. Herbert C. Friedmann for critically reading the manuscript.

* This work was supported by a grant from the National Institutes of Health, U.S. Public Health Service. A preliminary report has appeared: H. Wiesmeyer and M. B. Yarmolinsky, *Bacteriol. Proc.*, P120 (1960).

The following abbreviations are used: TPN, triphosphopyridine nucleotide; TPNH, reduced triphosphopyridine nucleotide; DPN, diphosphopyridine nucleotide; UDPG, uridine diphosphoglucose; UDPGal, uridine diphosphogalactose; G-1-P, α-D-glucose-1-phosphate; Gal-1-P, α-D-galactose-1-phosphate; UTP, uridine triphosphate; PP, pyrophosphate; *o*NPG, *o*-nitrophenyl-β-D-galactoside; *o*NP, *o*-nitrophenol.

† Post-doctoral fellow of the National Science Foundation.

‡ Contribution No. 305 of the McCollum-Pratt Institute.

[1] Morse, M. L., E. M. Lederberg, and J. Lederberg, *Genetics*, **41**, 142 (1956).

[2] Arber, W., G. Kellenberger, and J. Weigle, *Schweiz. Z. allg. Path. Bakt.*, **20**, 659 (1957).

[3] Arber, W., *Arch. Sci. (Geneva)*, **11**, 259 (1958).

[4] Campbell, A., *Virology*, **4**, 366 (1957).

[5] Buttin, G., F. Jacob, and J. Monod, *C. R. Acad. Sci., Paris*, **250**, 2471 (1960).

[6] Morse, M. L., E. M. Lederberg, and J. Lederberg, *Genetics*, **41**, 748 (1956).

[7] Kaiser, A. D., and F. Jacob, *Virology*, **4**, 509 (1957).

[8] Appleyard, R. K., *Genetics*, **39**, 440 (1954).

[9] Campbell, A., and E. Balbinder, *Genetics*, **44**, 309 (1959).

[10] Weigle, J., *Virology*, **4**, 14 (1957).

[11] Jacob, F., C. Fuerst, and E. Wollman, *Ann. Inst. Pasteur*, **93**, 724 (1957).

[12] Arber, W., and G. Kellenberger, *Virology*, **5**, 458 (1958).

[13] Lwoff, A., *Bacteriol. Revs.*, **17**, 269 (1953).

[14] Monod, J., and M. Cohn, in *Advances in Enzymology*, ed. F. F. Nord (New York: Interscience, 1952), vol. 13, p. 67.

[15] Jacob, F., and A. Campbell, *C. R. Acad. Sci., Paris*, **248**, 3219 (1959).

[16] Pardee, A. B., F. Jacob, and J. Monod, *J. Mol. Biol.*, **1**, 165 (1959).

[17] Kalckar, H. M., K. Kurahashi, and E. Jordan, these PROCEEDINGS, **45**, 1776 (1959).

[18] Kurahashi, K., *Science*, **125**, 114 (1957).

[19] Lederberg, E. M., in *Microbial Genetics* (Cambridge University Press, 1960), p. 115.

[20] Jordan, E., unpublished work in this laboratory.

[21] Zinder, N., *Virology*, **5**, 291 (1958).

[22] The method of D. K. Fraser (personal communication) is as follows: Cells lysogenic for

lambda are grown for three hours on tryptone broth in the presence of λimm^{434} and survivors are plated. Among them a high proportion will now be sensitive to lambda.

[23] Lederberg, J., and E. M. Lederberg, *Genetics*, **38**, 51 (1953).

[24] Kaiser, A. D., *Virology*, **3**, 42 (1957).

[25] Jacob, F., and E. L. Wollman, *Ann. Inst. Pasteur*, **91**, 486 (1956).

[26] Wollman, E. L., *Ann. Inst. Pasteur*, **84**, 281 (1953).

[27] Lederberg, J., *J. Bact.*, **56**, 695 (1948).

[28] Lwoff, A., L. Siminovitch, and N. Kjelgaard, *Ann. Inst. Pasteur*, **79**, 815 (1950).

[29] Kaiser, A. D., *Virology*, **4**, 424 (1955).

[30] Adams, M. H., *Bacteriophages* (New York: Interscience, 1959).

[31] Roberts, R. B., D. B. Cowie, P. H. Abelson, E. T. Bolton, and R. J. Britten, *Studies of Biosynthesis in Escherichia coli* (Carnegie Inst. of Washington, Publ. 607, Washington, 1957).

[32] Kaiser, A. D., and D. S. Hogness, *J. Mol. Biol.*, in press. The composition of the diluent is 0.01 *M* potassium phosphate buffer, pH 7.0, 0.01 *M* $MgSO_4$, and 10 μg per ml of bovine plasma albumin.

[33] Séchaud, J., and E. Kellenberger, *Ann. Inst. Pasteur*, **90**, 102 (1956).

[34] Najjar, V. A., in *Methods in Enzymology*, ed. S. P. Colowick and N. O. Kaplan (New York: Academic Press, 1955), vol. 1, p. 294.

[35] Kornberg, A., and B. L. Horecker, in *Methods in Enzymology*, ed. S. P. Colowick and N. O. Kaplan (New York: Academic Press, 1955), vol. 1, p. 323.

[36] Leloir, L. F., and A. C. Paladini, in *Methods in Enzymology*, ed. S. P. Colowick and N. O. Kaplan (New York: Academic Press, 1957), vol. 3, p. 143.

[37] Maxwell, E. S., *J. Biol. Chem.*, **229**, 139 (1957).

[38] Munch-Petersen, A., H. M. Kalckar, and E. E. B. Smith, *Kgl. Danske Videnskab. Selskab Biol. Medd.*, **22**, 3 (1955).

[39] Lederberg, J., *J. Bact.*, **60**, 381 (1950).

[40] Horecker, B. L., and P. Z. Smyrniotis, *J. Biol. Chem.*, **193**, 371 (1951).

[41] Kurahashi, K., and A. Sugimura, *J. Biol. Chem.*, **235**, 940 (1960).

[42] Kurahashi, K., personal communication.

[43] Zamenhof, S., in *Methods in Enzymology*, ed. S. P. Colowick and N. O. Kaplan (New York: Academic Press, 1957), vol. 3, p. 702.

[44] Jacob, F., and J. Monod, *C. R. Acad. Sci., Paris*, **249**, 1282 (1959).

[45] Jacob, F., D. Perrin, C. Sanchez, and J. Monod, *C. R. Acad. Sci., Paris*, **250**, 1727 (1960).

[46] Vogel, H. J., these PROCEEDINGS, **43**, 491 (1957).

[47] Kalckar, H. M., B. Braganca, and A. Munch-Petersen, *Nature*, **172**, 1038 (1953).

[48] Gale, E. F., and J. P. Folkes, *Biochem. J.*, **53**, 493 (1953).

[49] Bessman, M., personal communication.

[50] Matsushiro, A., and K. Mizobuchi, *Jap. J. Genetics*, **34**, 282 (1959).

[51] Starlinger, P., personal communications cited in references 5 and 50.

[52] Kalckar, H. M., and E. Jordan, *Science*, **131**, 1318 (1960).

[53] Puck, T. T., and H. H. Lee, *J. Exp. Med.*, **99**, 481 (1954).

[54] Jacob, F., and E. L. Wollman, *Ann. Inst. Pasteur*, **95**, 497 (1958).

INVARIANT IMBEDDING AND THE REDUCTION OF TWO-POINT BOUNDARY VALUE PROBLEMS TO INITIAL VALUE PROBLEMS

By Richard Bellman, Robert Kalaba, and G. Milton Wing

THE RAND CORPORATION, SANTA MONICA, CALIFORNIA

Communicated by S. Chandrasekhar, October 25, 1960

1. *Introduction.*—In a long series of papers (cf. ref. 1 for an extensive bibliography), we have applied the method of invariant imbedding to a wide variety of transport problems. The method characteristically replaces the two-point boundary value problems arising from the classical transport formulation by initial value problems. In most of our work, interaction between moving particles has not been allowed. The classical formulation is then linear, and it has been realized[2] that the invariant imbedding method provides a way of reducing systems of linear differential equations subject to boundary conditions at two points to simple initial value problems for nonlinear systems. In a few instances, relatively special cases of particle-particle interactions were allowed.[3, 4] The resulting invariant imbedding analyses were always somewhat involved.

In this paper, the strength of the imbedding method as applied to the reduction of quite general nonlinear systems of differential equations is more fully recognized. We present a heuristic derivation of our results, showing how such a system may be considered as representing mathematically a classical transport problem and then proceeding formally to the invariant imbedding analysis of the physical problem. A partial connection with classical characteristic theory is indicated, and a few examples are given.

2. *The Formal Results.*—Consider the system

$$\begin{aligned}
\frac{du}{dz}(z,r) &= F(u(z,1), \ldots, u(z,N), v(z,1). \ldots, v(z,N),z;r), \\
-\frac{dv}{dz}(z,r) &= G(u(z,1), \ldots, u(z,N), v(z,1), \ldots, v(z,N),z;r), \\
u(0,r) &= w_r, v(x,r) = y_r, r = 1,2, \ldots, N. \qquad (2.1)
\end{aligned}$$

The discrete version of (2.1) is

$$\begin{aligned}
u(z + \Delta,r) &= u(z,r) + F(u(z,i), v(z,j), z;r)\Delta + o(\Delta), \\
v(z) &= v(z + \Delta,r) + G(u(z,i), v(z,j), z;r)\Delta + o(\Delta), \qquad (2.2)
\end{aligned}$$

where $F(u(z,i), v(z,j), z;r) = F(u(z,1), \ldots, u(z,N), v(z,1,), \ldots, v(z,N), z;r)$, and so on.

Equations (2.2) may be interpreted as follows. Let particles move on a rod extending from 0 to x. Let the flux of particles in state r and moving to the right at z be denoted by $u(z,r)$. Let there be an interaction such that in going from z to $z + \Delta$, the flux is changed by an amount F. Both particle-medium and particle-particle interactions are permissible. A similar interpretation of v as a left-moving flux is obvious. An input of $\{y_r\}$ particles in state r ($r = 1, 2, \ldots, N$) impinges from the right at $z = x$, one of w_r from the left at $z = 0$.

With this physical model in mind, we define $R(z,r;y_1,y_2, \ldots, y_N)$ as the flux of

particles emerging from the right end of a rod of length x when the input is as described in the previous paragraph. It is clear that

$$u(x,r) \equiv R(x,r;y_1,y_2, \ldots, y_N). \tag{2.3}$$

FIG. 1.—The physical situation

Applying the functional equation method of invariant imbedding, we obtain the relation

$$R(x + \Delta, r;y,y_2, \ldots, y_N) = R(x,r; v(x,1), \ldots, v(x,N)) + F(u(x,i), v(x,j), x;r)\Delta + o(\Delta). \tag{2.4}$$

The physical interpretation of (2.4) is quite easy. The flux of particles out of a rod of length $x + \Delta$ consists of two parts: the flux out of the rod of length x due to an imput from the y_r's modified by the interaction in $(x,x + \Delta)$, plus the right-moving flux arising from this interaction.

From (2.2), we get

$$v(x,r) = y_r + G(R(x,i;y_1, \ldots, y_N), y_j, x;r)\Delta + O(\Delta). \tag{2.5}$$

Using this in (2.4) and letting $\Delta \to 0$, we obtain

$$\frac{\partial R}{\partial x}(x,r;y_1, \ldots, y_N) - \sum_k G(R(x,i,y_1, \ldots, y_N), y_j, x;k)\frac{\partial R}{\partial y_k} = F(R(x,i; y_1, \ldots, y_N),y_j, x;r). \tag{2.6}$$

Clearly,

$$R(0,r;y_1, \ldots, y_N) = w_r, r = 1,2, \ldots, N. \tag{2.7}$$

It now suffices to solve (2.6). One thus obtains, using (2.3), the values of $u(x,r)$, $r = 1,2, \ldots, N$. Once these are known, (2.4) becomes an initial value problem.

We may now define $T(x,r;y_1,y_2, \ldots, y_N)$ as the flux of particles in state r transmitted through the rod of length x, so that

$$v(0,r) \equiv T(x,r;y_1, \ldots, y_N). \tag{2.8}$$

Then,

$$T(x + \Delta,r;y_1, \ldots, y_N) = T(x,r;v(x,1), \ldots, v(x,N)) + o(\Delta), \tag{2.9}$$

and so,

$$\frac{\partial T}{\partial x} - \sum_k G(R(x,i;y_1, y_2, \ldots, y_N), y_j, x;k)\frac{\partial T}{\partial y_k} = 0, \tag{2.10}$$

with

$$T(0,r;y_1,y_2, \ldots, y_N) = y_r, r = 1,2, \ldots, N.$$

It is likewise easy to write down an equation for the dissipation function.[5] Further, the viewpoint may now be changed, and R and T as functions of the inputs

w_r at zero may be analyzed. One may then pick various equations to describe the process and utilize the set of equations which seems most convenient for the problem at hand.

3. *Connection with Classical Characteristic Theory.*—The solutions u to the system (2.1) may be considered as functions of the v's.

$$u(z,r) \equiv U(z,r;v(z,1)v(z,2), \ldots, v(z,N)). \tag{3.1}$$

Hence, in an obvious notation,

$$\begin{aligned} \frac{dU}{dz} &= \frac{\partial U}{\partial z} + \sum_k \frac{\partial U}{\partial v_k} \frac{dv(z,k)}{dz} \\ &= \frac{\partial U}{\partial z} - \sum_k \frac{\partial U}{\partial v_k} G_k. \end{aligned} \tag{3.2}$$

At $z = x$, this gives (2.6).

The original equations (2.1) are the equations of the characteristics of equation (2.6), the equation for the reflection function.

4. *Some Examples.*—Suppose (2.1) is simply

$$\frac{du}{dz} = f(z)v, \; -\frac{dv}{dz} = g(z)u, \; u(0) = 0, \; v(x) = y. \tag{4.1}$$

Then, (2.6) becomes

$$\frac{\partial R}{\partial x}(x,y) - g(x)R \frac{\partial R}{\partial y} = f(x)y. \tag{4.2}$$

The linearity of (4.1) suggests setting

$$R(x,y) = y\bar{R}(x), \tag{4.3}$$

and (4.2) reduces to the Riccati equation,

$$\frac{d\bar{R}}{dx} - g(x)\bar{R}^2 = f(x), \; \bar{R}(0) = 0. \tag{4.4}$$

The case $f = g = 1$ has been encountered early in our work.[6] There,

$$\frac{d\bar{R}}{dx} = \bar{R}^2 + 1. \tag{4.5}$$

Another problem studied previously in our consideration of neutron-neutron interactions[3] leads to equations with

$$F = v - buv, \; G = u - buv. \tag{4.6}$$

Equation (2.6) then yields

$$\frac{\partial R}{\partial x} = y - byR + R \frac{\partial R}{\partial y}(1 - by), \tag{4.7}$$

a result obtained much less easily in reference 3.

5. *Concluding Remarks.*—Our presentation here has been quite heuristic. A rigorous exposition will appear elsewhere. It should be noted that singularities may be encountered in integrating (2.6) even though the solution to (2.1) may be well-behaved. Thus (4.5) becomes singular at $x = \pi/2$, although the corresponding solutions u and v exist provided $x \neq n\pi/2$, $n = 1,2, \ldots$. Methods of continuing the solution of (2.6) will be considered in subsequent publications.

Once the equations for R, T, and D have been established on a firm basis, these functions may be used to establish existence and uniqueness theorems for systems of the kind (2.1)(cf. ref. 4).

It is reasonable to suspect that the ideas presented here can be expanded to more general functional equations such as differential-difference equations and integro-differential equations. Problem (4.1) suggests that an investigation of Sturm-Liouville problems using the R-function can be made. These matters will be discussed subsequently.

[1] Bellman, R., R. Kalaba, and G. M. Wing, "Invariant Imbedding and Mathematical Physics—I: Particle Processes," *J. Math. Physics*, **1**, 280–308 (1960).

[2] Bellman, R., and R. Kalaba, "Transport Theory and Invariant Imbedding," *Proc. Amer. Math. Soc. Symp. on Reactor Theory* (to appear).

[3] Bellman, R., R. Kalaba, and G. M. Wing, "Invariant Imbedding and Neutron Transport Theory—III: Neutron-Neutron Collision Processes," *J. Math. and Mech.*, **8**, 249–262 (1959).

[4] Bellman, R., R. Kalaba, and G. M. Wing, "Invariant Imbedding, Conservation Relations, and Nonlinear Equations with Two-Point Boundary Values," these PROCEEDINGS, **46**, 1258–1260 (1960).

[5] Bellman, R., R. Kalaba, and G. M. Wing, "Dissipation Functions and Invariant Imbedding," these PROCEEDINGS, **46**, 1145–1147 (1960).

[6] Bellman, R., R. Kalaba, and G. M. Wing, "On the Principle of Invariant Imbedding and Neutron Transport Theory—I: One-Dimensional Case," *J. Math. and Mech.*, **7**, 149–162 (1958).

---

*TESTS OF FORM OF THE ONE-PION EXCHANGE POTENTIAL**

BY G. BREIT, M. H. HULL, JR., K. E. LASSILA, AND H. M. RUPPEL

YALE UNIVERSITY

Communicated October 3, 1960

The one-pion exchange potential (OPEP) has been used in calculations on nucleon-nucleon scattering by various authors.[1–4] Its mathematical form has apparently remained untested so far.[5] This note is a report on some tests which indicate that an approximately correct value for the long-distance part of the potential is given by the pseudoscalar meson theory. The possibility of making the test suggested itself by the fact that the pion-nucleon coupling constant can be determined with reasonable accuracy by employing the OPEP in the calculation of phase parameters for large values of the orbital angular momentum $L\hbar$ and adjusting the magnitude of the potential as a whole through a variation of the coupling parameter g^2. It appeared possible, therefore, that the collection of nucleon-nucleon scattering

data at different energies may be adequate for the determination of coefficients of parts of the expression for the one-pion exchange potential,[6]

$$V^{(2)} = \frac{g^2}{3}\left(\frac{\hbar\mu}{2Mc}\right)^2 (\boldsymbol{\tau}_1\cdot\boldsymbol{\tau}_2)[(\boldsymbol{\sigma}_1\cdot\boldsymbol{\sigma}_2) + S_{12}\ (1 + \frac{3}{x} + \frac{3}{x^2})]\frac{e^{-\mu r}}{r}, \quad (1)$$

where $\boldsymbol{\tau}_1$, $\boldsymbol{\tau}_2$ are the isotopic spin vectors of the nucleons written in the same convention as the Pauli spin vectors $\boldsymbol{\sigma}_1$, $\boldsymbol{\sigma}_2$, $\mu = m_\pi c/\hbar$ where m_π is the pion mass, $x = \mu r$ where r is the internucleon distance, S_{12} is the usual tensor-force operator, and $g^2 = 137.0\ e^2g_0^2 = \hbar c g_0^2$ determines the coupling constant ≈ 14.

Methods Used.—The expression for $V^{(2)}$, as in equation (1), is of the form

$$V^{(2)} = V_{\boldsymbol{\sigma}\cdot\boldsymbol{\sigma}}{}^{(2)} + V_T{}^{(2)}, \quad (2)$$

the two parts on the right hand side referring to the spin-spin and the tensor parts of the potential. The first-order effects on the phase shifts corresponding to the potential can also be broken into parts, these corresponding respectively to the spin-spin and the tensor parts of the potential. The phase-parameters thus have the form

$$\delta^{1\pi} = \delta^{1\pi}(\boldsymbol{\sigma}\cdot\boldsymbol{\sigma}) + \delta^{1\pi}(T) \quad (3)$$

in the same notation as in equation (2). Three variations on this expression were used as follows:

$$\delta_a = \delta^{1\pi} + q_a\delta^{1\pi}(\boldsymbol{\sigma}\cdot\boldsymbol{\sigma}) = (g_0^2/14)[\delta_0{}^{1\pi} + q_a\delta_0{}^{1\pi}(\boldsymbol{\sigma}\cdot\boldsymbol{\sigma})] \quad (3.1)$$

$$\delta_b = \delta^{1\pi} + (14/g_0^2)q_b\delta^{1\pi}(\boldsymbol{\sigma}\cdot\boldsymbol{\sigma}) = (g_0^2/14)\delta_0{}^{1\pi} + q_b\delta_0{}^{1\pi}(\boldsymbol{\sigma}\cdot\boldsymbol{\sigma}) \quad (3.2)$$

$$\delta_c = \delta^{1\pi} + (14/g_0^2)q_c\delta_c{}^{1\pi} = \delta^{1\pi} + q_c\delta_{c0}{}^{1\pi} \quad (3.3)$$

where $\delta_0{}^{1\pi}$, $\delta_0{}^{1\pi}(\boldsymbol{\sigma}\cdot\boldsymbol{\sigma})$, $\delta_{c0}{}^{1\pi}$ are respectively the values of $\delta^{1\pi}$, $\delta^{1\pi}(\boldsymbol{\sigma}\cdot\boldsymbol{\sigma})$, and $\delta_c{}^{1\pi}$ for the standardized value $g_0^2 = 14$ and $\delta_c{}^{1\pi}$ is the first-order phase shift corresponding to the central potential $q_cV_c{}^{(2)}$ with

$$V_c{}^{(2)} = \frac{1}{3}g^2\left(\frac{\hbar\mu}{2Mc}\right)^2 e^{-\mu r}/r. \quad (3.4)$$

This is the potential which would be obtained for $(\boldsymbol{\tau}_1\cdot\boldsymbol{\tau}_2) = 1$ and $(\boldsymbol{\sigma}_1\cdot\boldsymbol{\sigma}_2) = 1$ and omitting S_{12}. For it to the first order in g^2,

$$\delta_c = -(1/6)(g^2k/4E_{1\text{cm}})(\mu^2/k^2)Q_L(1 + a^2/2), \quad (3.5)$$

with

$$a = \mu/k. \quad (3.6)$$

Here $E_{1\text{ cm}} \cong Mc^2$ is the relativistic kinetic energy of one nucleon in the center of mass system including the rest-mass energy. The central potential in terms of which the comparison is made is $-(1/3)$ of the singlet-even potential obtainable from equation (1) by setting $(\boldsymbol{\tau}_1\cdot\boldsymbol{\tau}_2) = 1$, $(\boldsymbol{\sigma}_1\cdot\boldsymbol{\sigma}_2) = -3$.

The tests to be described consist in the determination of reasonable upper limits for q_a, q_b, and q_c on the basis of existing experimental evidence. The constant q_a measures the fractional change in the spin-spin part of the OPEP that is being admitted. The parameter q_b has nearly the same significance. The difference between them is seen by comparing equations (3.1) and (3.2). If the knowledge of g_0

were certain, there would clearly be no value in introducing two such parameters. Since, however, g_0^2 is not known with certainty, it was varied also in fitting the data. By arranging for the two forms δ_a and δ_b, the limits on the final upper and lower limits are arrived at in different ways, but the ultimate objective is the same in the two cases. For perfect fitting work one would expect

$$g_0^2 q_a = 14 q_b. \tag{3.7}$$

Since perfect fitting in the present case implies also perfect assignment of error limits, one cannot expect this equation to be accurately satisfied by the upper and lower limits of q_a and q_b. The convention used in equation (3.3) is to add the effect of the central potential in terms of (3.4) with the value of g_0^2 fixed arbitrarily at 14.

In phase-parameter fits[3-5] to p-p and n-p data, some phase parameters have been "searched" and some have been used with values corresponding to the OPEP. The variations of g_0^2 and of the q were studied for the OPEP phase parameters, the searched parameters being kept fixed at the values determined by the general search. The mean weighted sum of the squares of the deviations,[7] D, was plotted against g_0^2 and against one of the q's for each of the three cases (a), (b), (c) represented respectively by equations (3.1), (3.2), (3.3). Thus, in case (a), a family of curves representing D versus g_0^2 is obtained, each curve corresponding to one q_a. Similarly, another family of curves is obtained for fixed g_0^2 with D plotted against q_a. The values of D at the minimum and of the abscissae were read and the variations of g_0^2 and q_a needed to produce a change of D by $\pm 1/N$ from its minimum value were read also. Here N is the number of measurements used in the analysis. In this way, the first family of curves gives for each q_a the most probable g_0^2 and a measure of the uncertainty in its value. Similarly, the second family gives for assigned g_0^2 the most probable q_a and uncertainties in its value.

The error estimates depend appreciably on whether the problem is regarded as that of the determination of one or of several parameters. If, for example, the value of g_0^2 were supposed known, then only one of the curves just described for the fixed g_0^2 would be needed. If, on the other hand, the possibility of g_0^2 having an adjustable value is admitted and no other information is used than that contained in the adjustment to scattering data, then the accuracy in the determination of q is affected by the uncertainty in the value of g_0^2. Denoting one of the variables to be determined by $x + x_0$ and the other by $y + y_0$ one has, close to the minimum of D,

$$D - D_{\min} = D' = \alpha x^2 + 2\beta xy + \gamma y^2 \tag{4}$$

with x_0 and y_0 taken to be the values of the original variables for the minimum of D. By an orthogonal transformation of (x, y) to (x', y'), the quadratic form can be brought into a form

$$D' = \alpha' x'^2 + \gamma' y'^2 \tag{4.1}$$

involving only squares which indicates uncorrelated errors for x' and y'. Estimating $Nx'^2 = 1/\alpha'$, with N standing for the number of observations, it follows after a simple calculation that

$$N(\langle x^2 \rangle, \langle y^2 \rangle, \langle xy \rangle) = (\alpha\gamma - \beta^2)^{-1}(\gamma, \alpha, -\beta). \tag{4.2}$$

The correlation coefficient will not be of immediate interest below. The values of $\langle x^2 \rangle$ and $\langle y^2 \rangle$ can also be obtained in the form just stated by an easy calculation for a two-dimensional Gauss error distribution. In this case, if an integration of the two-dimensional distribution is performed over y, the remaining one-dimensional distribution over x is normal and has a standard deviation $\langle x^2 \rangle^{1/2}$ with $\langle x^2 \rangle$ as in equation (4.2). The relationship of the parameters of the two-dimensional distribution to (α, β, γ) becomes apparent in the diagonal form of the quadratic in the exponent of the Gauss error distribution and, by transformation to original axes, gives the Gauss error distribution for which the integration over y has been just mentioned.

It is convenient to consider the lines

$$\partial D'/\partial x = 0, \qquad \partial D'/\partial y = 0 \tag{4.3}$$

in the (x, y) plane. They correspond respectively to minima of D for variation with respect to x and y. In the approximation of equation (4), the loci of these minima are

$$^1/_2 \partial D'/\partial x \cong \alpha x + \beta y = 0, \qquad ^1/_2 \partial D'/\partial y \cong \beta x + \gamma y = 0, \tag{4.4}$$

but in general the loci are curves. In the rectilinear approximation, two cases are of interest as follows: (A) If one moves along $\partial D'/\partial y = 0$ up to $D' = 1/N$ from $D' = 0$, then x changes from 0 to x_A such that

$$N x_A^2 = \gamma/(\alpha\gamma - \beta^2), \tag{4.5}$$

as is readily verified by means of (4.4) and (4). The right-hand side of (4.5) is the same as the first of the three numbers on the right-hand side of (4.2), so that $x_A^2 = \langle x^2 \rangle$, showing that the $1/N$ relationship can be used by moving along the line of minima of D with respect to y variation. This relation shows geometrically the difference between $\langle x^2 \rangle$, following from a two-dimensional study of q, and g_0^2, as compared with its value for an assigned g_0^2. (B) In the latter case, assuming that y_0 has been properly chosen, one has to move along $y = 0$, and then the value $1/N$ is reached for

$$N x_B^2 = 1/\alpha. \tag{4.6}$$

Similarly, by moving along $x = 0$ to a point for which $D' = 1/N$, there is obtained a y_B such that

$$N y_B^2 = 1/\gamma. \tag{4.7}$$

Plotting the curves (4.3), one has available through their slopes the approximations (4.4) and hence also the ratios $\alpha:\beta$ and $\beta:\gamma$. These combined with (4.6) and (4.7) overdetermine α, β, γ, making a check possible. Employment of (4.6) and (4.7) is much simpler than manipulations with x_A^2, y_A^2 would be. Introducing

$$(A, B, C) = N(\alpha, \beta, \gamma), \tag{5}$$

the equations giving the quantities of main interest are

$$(\langle x^2 \rangle, \langle y^2 \rangle, \langle xy \rangle) = (AC - B^2)^{-1}(C, A, -B), \tag{5.1}$$

$$\Delta q \equiv x_B = A^{-1/2}, \qquad \Delta(g_0^2) \equiv y_B = C^{-1/2}. \tag{5.2}$$

If the value of g_0^2 were known and were to correspond to $y = 0$, the standard deviation of q would be Δq, and if q were known to be zero, then the uncertainty in the g_0^2 would be Δg_0^2. Since $D > 1$, the assumption regarding the standard deviations of the experimental points being correctly given by the errors stated in the experimental papers is unlikely to be correct and, therefore, all error limits calculated by means of equations (5.1), (5.2) are multiplied below by $\sqrt{D}$.

Results Obtained.—Data on p-p scattering employing a preliminary form of fit[8] YLAM gave for assumed values of g_0^2 values of q_a, q_b, q_c as in Table 1. The parameters which were not varied as OPEP phase parameters were all those for $J \leq 3$ and in addition ${}^{\theta}F_4$, ρ_4 and K_4. In the preliminary form of the YLAM fit used for the tests reported on, the values of K_4 were first fixed at their OPEP values but were searched for best fit to data and the searched values were used. The fixing of K_4 at its OPEP value for the final YLAM fit occurred later.

TABLE 1

VALUES OF PARAMETERS q_a, q_b, q_c AS DETERMINED FROM SEARCH YLAM OF p-p SCATTERING DATA

Assumed g_0^2	q_a	q_b	q_c
12	-0.14 ± 0.18	-0.12 ± 0.15	0.19 ± 0.30
14	-0.21 ± 0.16	-0.22 ± 0.15	-0.09 ± 0.27
17	-0.32 ± 0.13	-0.36 ± 0.16	-0.48 ± 0.34

The numbers following the $\pm$ in the table are the Δq determined by means (5.2) and multiplied by $D^{1/2}$, the minimum D for the q variation being used. The test by means of equation (3.7) indicates that the last digit in q is not reliable, as is natural since the location of the minimum of D on the D-versus-q plots is subject to graphical inaccuracies. Even though errors of a few units in the last digit are possible, they are definitely less than the statistical errors listed. If g_0^2 is taken to be known on the basis of other information, then the values of q_a, q_b, q_c following from p-p scattering on the basis of assumptions used are those in Table 1. From the same data and the same form of search YLAM, the values of g_0^2 for assumed values of q_a, q_b, q_c are listed in Table 2.

TABLE 2

VALUES OF g_0^2 AS DETERMINED FROM SEARCH YLAM OF p-p SCATTERING DATA AND ASSUMED VALUES OF q_a, q_b, q_c

q_a	q_b	q_c	g_0^2
0	0	0	13.6 ± 1.2
$-1/4$	0	0	14.4 ± 1.2
$1/4$	0	0	12.5 ± 1.1
0	$-1/4$	0	14.2 ± 1.2
0	$1/4$	0	12.7 ± 1.2
0	0	$-1/2$	14.6 ± 1.2
0	0	$1/2$	12.4 ± 1.1

According to Table 2, the value 14 for g_0^2 is reasonably close to the most probable if it is assumed that the $q = 0$. According to Table 1 for this g_0^2 the difference of the q from zero is barely in excess of the error of the determination. Table 1 shows that q_a, q_b and q_c are sensitive to the assumed g_0^2, and Table 2 indicates some sensitivity of g_0^2 to the assumed values of the q. Without bringing in other data, it is, therefore, necessary to make use of equation (5.1) in a simultaneous treatment of

the statistical uncertainties. This has been done by plotting the values of q_a corresponding to minima of D for fixed g_0^2 against the g_0^2 used together with values of $q_a \pm \Delta q_a$ giving three approximate curves. The values in Table 1, together with values for additional g_0^2, were used for these graphs. On the same sheet, the values of g_0^2 corresponding to the minimum of D for fixed q_a and variable g_0^2 were plotted together with roughly parallel graphs for $g_0^2 \pm \Delta(g_0^2)$. The intersection of the center graphs of each set of three gives the most probable pair of g_0^2 and q_a. The four outer curves of both sets form a parallelogram-like figure within which the more probable values of x and y are located. Representing the figure by a parallelogram, the approximation of equation (4.4) is obtained, and the possibility of approximating the figure in this manner justifies the employment of equations (5.1), (5.2) in most cases considered. The same procedure was used for q_b and q_c. In Table 3, the values so obtained are summarized.

TABLE 3

VALUES OF (q, g_0^2) PAIRS FROM p-p SEARCH YLAM WITH ERRORS CALCULATED BY EQUATION (5.1) AND MULTIPLIED BY $D^{1/2}$

Case	q	g_0^2
a	-0.24 ± 0.16	14.0 ± 1.3
b	-0.22 ± 0.17	14.1 ± 1.3
c	-0.04 ± 0.33	13.6 ± 1.4

If the errors are estimated on the basis of extreme values in the curvilinear parallelogram, again with inclusion of factor $D^{1/2}$, they are $+0.26$, -0.33 for the first entry of second column, ± 0.25, ± 0.64 for the second and third entries, and ± 1.4, ± 2.0, and ± 2.6 for the first, second, and third rows of the last column respectively. These values are probably very conservative estimates of the accuracy of the determination. But it would hardly be justifiable to conclude from Table 3 that the data indicate values of q_a, q_b, q_c significantly different from zero, especially if it is remembered that the graphical determination of the location of the minima is not quite accurate enough to determine the last digit, that a preliminary rather than a final set of phase parameters for search YLAM has been used, that the phase parameters of the non-one-pion category have appreciable statistical errors, and that the fit determined by YLAM, while most probably of the correct type, has not been established with absolute certainty. It appears more proper, therefore, to interpret Table 3 as indicating the absence of an outstanding deviation from the OPEP at distances that matter for the phase parameters that have been treated by means of this potential.

By a similar procedure, n-p data with fit[4,8] YLAN2M, employing variation of one-pion exchange parameters for $T = 0$ and 1 together, gave values as in Table 4, and for n-p data, fit[9] YLAN3, the values are as in Table 5. The parameters not varied as OPEP parameters were as mentioned for $T = 1$ in connection with Table 1 and for $T = 0$ all parameters with $J \leq 3$ and in addition $\delta^G{}_4$, $\theta^G{}_5$, and ρ. For $T = 0$, the phase parameters which were not included in the one-pion group were those for the states $^3S_1$, $^3D_1$, $^3D_2$, $^3D_3$, $^3G_3$, $^3G_4$, $^3G_5$, $^1P_1$, $^1F_3$, together with the coupling parameters ρ_1, ρ_3 and ρ_5. For $T = 1$, the phase parameters not included in the one-pion group were those for the states $^1S_0$, $^3P_0$, $^3P_1$, $^3P_2$, $^3F_2$, $^3F_3$, $^3F_4$, $^1D_2$, together with the coupling parameters ρ_2 and ρ_4. A later stage of YLAM was used in the present work for YLAN3 than for YLAN2M. The state $^1G_4$ was treated differently for $T = 0$ and $T = 0,1$ one-pion searches, the phase shift K_4 having been used as a one-pion

TABLE 4

VALUES OF (q, g_0^2) PAIRS DERIVED FROM n-p SEARCH YLAN2M WITH ERRORS CALCULATED BY EQUATION (5.1) AND MULTIPLIED BY $D^{1/2}$

Case	q	g_0^2
a	0.21 ± 0.21	13.1 ± 1.7
b	0.25 ± 0.17	12.5 ± 1.9
c	−1.0 ± 1.2	15.0 ± 1.6

TABLE 5

VALUES OF q AND g_0^2 DERIVED FROM n-p SEARCH YLAN3

Case	q	g_0^2	Values of T included in OPEP search
a	−0.14 ± 0.19	13.2 ± 1.7	0
	−0.27 ± 0.12	16.4 ± 1.6	0,1*
b	−0.15 ± 0.16	13.4 ± 1.8	0
	0.02 ± 0.14	12.0 ± 1.9	0,1*
c	−3.5 ± 2.6	13.3 ± 1.8	0
	−4.5 ± 1.0	11.8 ± 0.8	0,1*

* K_4 varied as a one-pion phase shift.

parameter for variations of T = 0,1 together and of T = 1, while for variations of the one-pion phase parameters of the T = 0 group alone, it naturally did not enter in the variations and kept at its one-pion value for $g_0^2 = 14$. Variations of the T = 1 group of one-pion phase parameters have also been made by themselves. The accuracy is somewhat poorer in this case than in the others reported on and, therefore, the values are not listed.

Varying g_0^2 alone for $q_a = q_b = q_c = 0$, there were obtained from n-p data with search YLAN3 values of g_0^2 as in Table 6.

TABLE 6

VALUES OF g_0^2 FROM n-p SEARCH YLAN3

Values of T for groups of one-pion phase parameters varied	0	1*	0,1*	0,1†
g_0^2	11.9 ± 1.2	11.9 ± 2.2	12.6 ± 0.9	12.5 ± 0.9

* K_4 varied with g_0.
† K_4 not varied with g_0.

For fit YLAN2M employing T = 0,1 with the g_0^2 variation and all $q = 0$, there was obtained the value $g_0^2 = 14.4 \pm 1.3$. Comparison of Tables 4 and 5 indicates that the average value of the q obtained from the phase-parameter sets YLAN2M and YLAN3 differs by amounts comparable with the statistical uncertainties. Fit YLAN3 represents the data quite well and may be reasonably close to the true one. However, in the case of ρ_1, there are some features which need improvement. Fit YLAN2M is open to the same objections as YLAN3 regarding ρ_1 to a somewhat greater extent. The evidence from n-p fits is doubtless not as certain as that from p-p fits. The large value of q_c listed in Table 5 may be partly attributable to this cause. But it appears probable that it is the result of the difference in the treatment of K_4, which was varied as an OPEP parameter for YLAN3 with T = 0, 1, combined, and with T = 1 alone (not displayed), but not in the other cases. Among the cases shown for YLAN3, T = 0,1 is the only one definitely calling for a q_c, the error of determination for YLAN3, T = 0 being comparable with the value. It requires perhaps further investigation to be absolutely sure that K_4 is sufficiently

different from its OPEP value to account for the large q_c and that one is dealing here with an indication of a real difference from the OPEP. The fact that q variations for the better-defined p-p fit, YLAM, in which K_4 was not varied as an OPEP parameter, give no definite indication of q_c also fits in with attributing the large absolute value of q_c in Table 5 to the treatment of K_4. This explanation of the large q_c is in agreement with the fact that the ${}^3G$ phase-shift parameters change by amounts comparable to their OPEP values in the YLAN3 search. It should also be observed that K_4, having the smallest L, is responsible for the largest contributions to the OPEP group. It is for this reason that the apparent accuracy of the determinations of q_c is high in the searches including K_4. The effect observed is of approximately the magnitude expected from other evidence since $-(1/3)\ (-4.5) = 1.5$, so that effectively the singlet even long-range potential apparently is roughly twice its OPEP value at the distances that matter most in this work.

On the whole, the results corroborate the tests of charge independence[4] which employed similar methods and the approximate estimates of the internucleon distances beyond which the OPEP may be assumed to be responsible for the major part of the nucleon-nucleon interaction,[6] the OPEP group of parameters being similar in the various sets of calculations. The procedure described above is obviously capable of furnishing more definite information regarding the localization of long-range deviations from the OPEP. Whether it can be carried much farther will depend partly on the accumulation of experimental data and on their accuracy. It is hoped that in future work it may prove possible to increase the accuracy of the statistical analysis regarding spatial localization by the employment of a systematic correlation between the L values of the phase parameters included in the varied OPEP group and the energy, as can be done through the employment of sensitivity curves of phase parameters to local changes in the potential.[6] It may prove possible to include other changes in the form of the added potential and in particular the range constants corresponding to the charged and neutral pion may perhaps be tested singly or in combination. The proportions with which they enter the $T = 0$ and $T = 1$ states for n-p scattering may then also serve as more definite indicators of whether g^2 or $g^2(m_\pi/2M)^2$ or possibly some other combination is the more fundamental coupling constant. Among extensions and improvements of the present work, there is the possibility of providing quantitative evidence regarding the two-pion exchange term which is furnished qualitatively in the present study. The absence of spin-spin and tensor term modifications and the presence of a central field term in the tests performed are in accordance with expectation for the two-pion exchange potential.

Summary.—The mathematical form of the one-pion exchange potential (OPEP) is tested by comparison with experimental material on nucleon-nucleon scattering. The tests are performed by modifying the form following from meson theory through changes in the proportions of spin-spin and tensor parts of the potential and also by adding a central potential part. These two tests are performed independently of each other, and in each case, the pion-nucleon coupling constant is allowed to vary simultaneously with the modifying parameter. Proton-proton and proton-neutron data are treated independently of each other. The experimental material is in the region from about 9 to 340 Mev. Within the statistical error of the determination of best values of the parameters, they are not significantly differ-

ent from zero as long as the conditions for the applicability of the OPEP are satisfied through exclusion of too low values of orbital angular momentum $L\hbar$. A definite deviation appears in tests of the central potential when $L = 4$ is admitted into the OPEP group of singlet even potentials as seen in Table 5. The absence of a definite effect for the proportion of spin-spin and tensor parts in tests performed fits expectation for the two-pion exchange potential.

* This research was supported by the U.S. Atomic Energy Commission under Contract AT (30-1)-1807 and by the Office of Ordnance Research, U.S. Army.

[1] Taketani, Nakamura, and Sasaki, *Progr. Theoret. Phys. (Kyoto)*, **6**, 581 (1951); Iwadare, Otsuki, Tamagaki, and Watari, *Progr. Theoret. Phys. (Kyoto)*, **16**, 455 (1956). No attempt to supply a complete bibliography is being made either in this or in the following footnotes, only the principal publications of each group of workers being mentioned. The choice otherwise has been governed by proximity of subject matter to the special phase discussed in the present paper.

[2] Moravcsik, Cziffra, MacGregor, and Stapp, *Bull. Amer. Phys. Soc.*, **4**, 49 (1959); Cziffra, MacGregor, Moravcsik, and Stapp, *Phys. Rev.*, **114**, 880 (1959).

[3] Breit, G., in *International Conference on Nuclear Forces and the Few Nucleon Problem*, University College, London, July 1959 (to be published). This report was based on work done in collaboration with M. H. Hull, Jr., K. D. Pyatt, Jr., C. R. Fischer, K. Lassila, and T. Degges.

[4] Breit, G., M. H. Hull, Jr., K. Lassila, and K. D. Pyatt, Jr., *Phys. Rev. Letters*, **4**, 79 (1960).

[5] The general possibility of doing so has been mentioned in Hull, Breit, Lassila, Pyatt, and Ruppel, *Bull. Amer. Phys. Soc.*, **5**, 268 (1960).

[6] The notation employed is the same as in G. Breit, *Phys. Rev.*, **111**, 652 (1958) with the change of f to g and in G. Breit and M. H. Hull, Jr., *Nuclear Phys.*, **15**, 216 (1960). The OPEP calculations referred to in the text have been made employing formulas in the last reference which are equivalent to those in ref. 2.

[7] The weighting factor is the reciprocal of the square of the standard deviation of an observation, and the mean is over all observations.

[8] Fits obtained by gradient searches employing as a starting point the phase parameters of Gammel, Christian, and Thaler, *Phys. Rev.*, **105**, 311 (1957) and of J. A. Gammel and R. M. Thaler, *Phys. Rev.*, **107**, 1337 (1957) supplemented by additional values kindly supplied by Messrs. Gammel and Thaler have designations with YLA for the first three letters. The YLAM set of fits is a modification of the original YLA referred to in refs. 3 and 4. Further details regarding YLAM are contained in G. Breit, M. H. Hull, Jr., K. E. Lassila, and K. D. Pyatt, Jr., "Phase-Parameter Representation of Proton-Proton Scattering from 9.7 to 345 Mev" (submitted for publication in the *Physical Review*). A similar designation of n-p fits with YLAN as the first four letters has been partly described in ref. 4.

[9] Fit YLAN3 is a development on fit YLAN2M with a major difference regarding the sign of ρ_1 through most of the energy range. It includes, among other matters, the employment of an improved mass treatment for the OPEP described in the last citation in ref. 8 (to be published) and differences in treatment of OPEP values for $^3F_3$, $^3F_4$, and ρ_4 at different states of the search.

A MODEL OF GROWTH AND GROWTH CONTROL IN MATHEMATICAL TERMS, II. COMPENSATORY ORGAN GROWTH IN THE ADULT†*

By J. Lee Kavanau

DEPARTMENT OF ZOOLOGY, UNIVERSITY OF CALIFORNIA AT LOS ANGELES

Communicated by Paul Weiss, October 24, 1960

Introduction.—The theory of growth and growth control advanced by Weiss[1] has been elaborated upon and formulated in mathematical terms by Weiss and Kavanau[2] in the first paper of this series. The resulting model transcends the more abstract models of the past in the closer approximation to reality of its basic premises, in its wider scope, which extends beyond a mere representation of growth to growth disturbances and regulations, and, above all, in the dissection of the growth problem into a series of detailed questions which lend themselves to experimental investigations. The model explicitly incorporates into its framework terms for the effects of differentiation, growth limitation by negative feedback, and catabolic loss as they are postulated to individually modify growth. Yet it refrains from introducing parameters that cannot be endowed with physical and biological meaning. By contrast, earlier treatments generally dealt only with the compound effect of factors affecting growth, representing this effect empirically as a single entity in formulations involving parameters of questionable or unassignable significance and unrealistic boundary value conditions. The present model, by virtue of its explicitness, has led to the elimination of several alternatives which proved to be unrealistic but whose inadequacy could not be ascertained by other means.

Additionally, this model represents a class of systems controlled by negative feedback, possessing such generality that in a formal sense it is equally valid for alternative biological or chemical systems, requiring for conversion merely simplification and/or a reassignment of significance of variables and parameters. For these reasons, investigations of its predictions, stability, and general behavior after disturbances of equilibrium take on a significance beyond that attached merely to its specific application to the problem of growth regulation in higher forms.

The basic assumptions upon which the model was predicated are

1. "Growth," as the measured gain of organic mass of a circumscribed living system, is the net balance of organic mass "produced" and retained over organic mass "destroyed" and otherwise lost.

2. The mass of an organic system is regarded as consisting of two functionally different components: (*a*) the generative mass, *G*, comprising the instruments of protoplasmic reproduction which remain in reproductive activity, and (*b*) the differentiated mass, *D*, derived from the former and consisting of terminal products and other secondary derivatives that do not possess the ability to reproduce.

3. Each specific cell type reproduces its protoplasm through the self-duplication and catalytic activity of key compounds ("templates") characteristic of the particular cell type. Each cell also produces specific freely diffusible compounds ("antitemplates") antagonistic to the former, which can block and thus inhibit the activity of the corresponding "templates."

4. The "antitemplates" act to regulate growth by a negative feedback mechanism in which their increasing populations render an increasing proportion of the homologous templates ineffective, resulting in a corresponding decline of the growth rate.

5. The attainment of terminal size is an expression of a stationary equilibrium between the incremental and decremental growth components and of the equilibration of the intracellular and extracellular antitemplate concentrations.

6. Both components of living systems, the generative mass and the differentiated mass (including the antitemplates), undergo continual metabolic degradation and replacement.

The model derived upon the basis of these assumptions could be expressed as three simultaneous differential equations involving several parameters of specified significance. Some of these parameters could be evaluated tentatively using experimental data for normal chicken growth. The resulting specific solution yielded a wholly reasonable representation of a growing organism.

Two parameters, relating to the daily catabolic loss of antitemplates (k_5) and the number of antitemplate particles required for the inactivation of a single template (x), cannot yet be specified. Instead, several reasonable values of these parameters were assumed and the resulting predictions for compensatory organ growth in the chicken determined. The model reproduced adequately both the compensatory spurt of growth of a tissue mass artificially reduced while within the overt growing stage and the automatic resumption of overt growth by the remainder of a mass reduced after having reached terminal size. The model also predicts spontaneous resumption of organ growth after artificial reduction of antitemplate concentration at terminal equilibrium. Quantitatively, however, the predicted reactions seemed too sluggish, but it was recognized that the sensitivity of the responding system would be greater for other values of the two parameters in question. In any case, the foregoing results represented a highly limited range of evaluation of the model and of the compensatory growth response, since they were determined manually by laborious stepwise numerical integration.

A more complete determination of the predictions of the model has now been carried out employing the IBM 709 Digital Computer[3] of the Western Data Processing Center of the University of California at Los Angeles. These computations have greatly extended our assessment of the model and of its responses. Upon the basis of the biological consistency of the resulting predictions, antitemplate production by the differentiated mass (one of two alternative sources which remained to be considered) has been ruled out. Also eliminated are certain ranges and combinations of values for the parameters x and k_5. Four parameter combinations were singled out for extensive study, leading to predictions for the behavior of adult organ systems after the imposition of several departures from steady-state equilibrium.

A brief résumé of the salient features associated with the formulation of the model is presented in the treatment below, together with the preliminary findings based upon manual calculations. These are followed by a formulation of the results obtained with the IBM 709 Computer. The latter results are discussed in terms of the predictions to which they lead, of the experimental determinations to which they point, and of their correlations with existing experimental findings.

Brief Résumé of the Formulation of the Model.—The differential equations given below express in words the theoretical structure of the model.

$$dG = \begin{bmatrix}\text{Change in}\\ \text{generative}\\ \text{mass}\end{bmatrix} = \underbrace{\begin{bmatrix}\text{Basic rate of}\\ \text{reproduction}\\ \text{of generative}\\ \text{mass}\end{bmatrix}\begin{bmatrix}\text{Generative}\\ \text{feedback}\\ \text{term}\end{bmatrix} dt}_{\text{Generative mass formed}} - \underbrace{\begin{bmatrix}\text{Initial rate}\\ \text{of conversion of}\\ \text{generative mass}\\ \text{into differentiated}\\ \text{mass}\end{bmatrix}\begin{bmatrix}\text{Differen-}\\ \text{tiation}\\ \text{feedback}\\ \text{term}\end{bmatrix} dt}_{\text{Generative mass lost}} \quad (1)$$

$$dD = \begin{bmatrix}\text{Change in}\\ \text{differentiated}\\ \text{mass}\end{bmatrix} = \underbrace{\begin{bmatrix}\text{Rate of gain of}\\ \text{differentiated mass}\\ \text{by conversion from G}\end{bmatrix} dt}_{\text{Differentiated mass produced}} - \underbrace{\begin{bmatrix}\text{Rate of catabolic}\\ \text{loss of differentiated}\\ \text{mass}\end{bmatrix} dt}_{\text{Differentiated mass lost}} \quad (2)$$

$$dI = \begin{bmatrix}\text{Change in number}\\ \text{of inhibitor}\\ \text{molecules}\end{bmatrix} = \underbrace{\begin{bmatrix}\text{Rate of}\\ \text{production of}\\ \text{inhibitors}\end{bmatrix} dt}_{\text{Inhibitors formed}} - \underbrace{\begin{bmatrix}\text{Rate of catabolic}\\ \text{loss of inhibitors}\end{bmatrix} dt}_{\text{Inhibitors lost}} \quad (3)$$

The selection of chicken growth data for guidance in the development of the model was based on their relative abundance, not on any advantage of the chicken for compensatory growth studies. In our initial efforts to formulate a model, a constant rate of differentiation of generative mass was assumed. However, actual calculations upon this basis produced values for the rate at which differentiated mass could be built up which were too low. Only if it were formed at a high rate during the period of rapid growth and at a declining rate as the rate of growth decreased, i.e., only if a progressive restraint were placed upon differentiation could a biologically adequate solution be formulated. This result was attained by placing the rate of differentiation also under negative feedback of the same origin as that limiting growth. This elaboration of the model was the first of several to emerge as a consequence of the explicitness of its formulation. It is noteworthy that the growth regulation predicted by the resulting differential equation for dG (Eq. 4) is compatible with the much-subscribed-to proposal of some degree of antagonism between growth and differentiation. Thus, it is predicted that growth is checked primarily by rapid differentiation during the period when its regulation by negative feedback is small. As differentiation diminishes in rate, the generative mass is gradually released from the sapping effect thereof, although this release tends to be

counteracted by the increasing feedback inhibition which progressively takes over as the growth regulating factor.

Several mechanisms for the production of antitemplates were envisioned: They might have been formed (*a*) continuously by existing differentiated mass ($D \rightarrow I$), or (*b*) by existing generative mass ($G \rightarrow I$), or (*c*) in the process of reproduction of generative mass, or (*D*) in the process of conversion of generative mass to differentiated mass (or by combinations of these mechanisms). Actual calculations (on the basis of normal chicken growth) led to the elimination of the alternatives (*c*) and (*d*). For both of these latter cases, the growth in volume of the "dilution pool" (the pool into which the antitemplates become distributed) would have to follow a patently absurd course. Both mechanisms (*a*) and (*b*), however, led to apparently reasonable values for growth of the dilution pool and were accordingly retained as alternatives for further consideration.

A general conclusion that could be drawn from considerations of the growth and relative volume of the dilution pool was that more than one antitemplate molecule is required for the inactivation of one template unless the rate of catabolic loss of antitemplates is lower than 0.10667 per day (half-life greater than $6\frac{1}{2}$ days). Further considerations of the growth curve for the dilution pool led to predictions in good agreement with the known relative increase in solid bulk during development as well as with the existence in the chick of an accessory fluid system in the extra-embryonic area prior to hatching.

The derived differential equations describing both the normal growth of the system and its behavior after experimental or pathological interference are

$$dG = G\left[\left[1 - \frac{b(I/I_e)^x}{(V/V_e)^x}\right]\log 2 - k_2 - k_1\left[1 - \frac{I/I_e)^x}{(V/V_e)^x}\right]\right]dt, \tag{4}$$

$$dD = \left[k_2G + k_1G\left[1 - \frac{(I/I_e)^x}{(V/V_e)^x}\right] - k_3D\right]dt, \tag{5}$$

$$dI = k_5I_e\left[\left|(G/G_e);(D/D_e)\right| - I/I_e\right]dt. \tag{6}$$

$$\text{restrictions:} \quad \left[1 - \frac{b(I/I_e)^x}{(V/V_e)^x}\right] \geq 0, \left[1 - \frac{(I/I_e)^x}{(V/V_e)^x}\right] \geq 0.$$

Normalizing, i.e., expressing *dG*, *dD*, and *dI* in units of the equilibrium values of *G*, *D*, and *I* respectively, the special cases of equations 4, 5, and 6 as applied to the adult are

$$dG = G(1 - bI^x)(\log 2)\,dt - G[k_2 + k_1(1 - I^x)]\,dt, \tag{4a}$$

$$dD = G[k_2 + k_1(1 - I^x)](k_2/k_2)\,dt - k_3D\,dt, \tag{5a}$$

$$dI = k_5[(G \text{ or } D) - I]\,dt. \tag{6a}$$

$$\text{restrictions:} \quad (1 - bI^x) \geq 0, (1 - I^x) \geq 0.$$

Preliminary Predictions Based Upon Manual Calculations.—Having ascertained the consistency of the model for normal chicken growth, it was next subjected to "experimental" tests; i.e., it was set to predict the behavior of organ systems whose normal growth or size was subject to experimental or pathological modifications.

The case of early compensatory growth following the removal of half of an adult

organ system, e.g., one kidney, was first considered (using an electric analogue computer). The results confirmed that the compensatory reaction would be far too sluggish if one antitemplate molecule sufficed for the inactivation of one template (inhibition proportional to the antitemplate concentration: $I \alpha C$). But they also showed that it would be too sluggish if a low rate of catabolic loss of antitemplates prevailed. Accordingly, in computing more accurate solutions manually, preference was given to cases entailing the inactivation of one template by two antitemplate molecules (inhibition proportional to the square of the antitemplate concentration: $I \alpha C^2$) with assumed antitemplate half-lives of $3^1/_4$ and $6^1/_2$ days.

Many of the resulting compensatory growth curves were found to have oscillatory character. They at first overshot the original (equilibrium) value, just as do many types of simple physical systems in their return to stable equilibrium after the disturbance of the stable state.

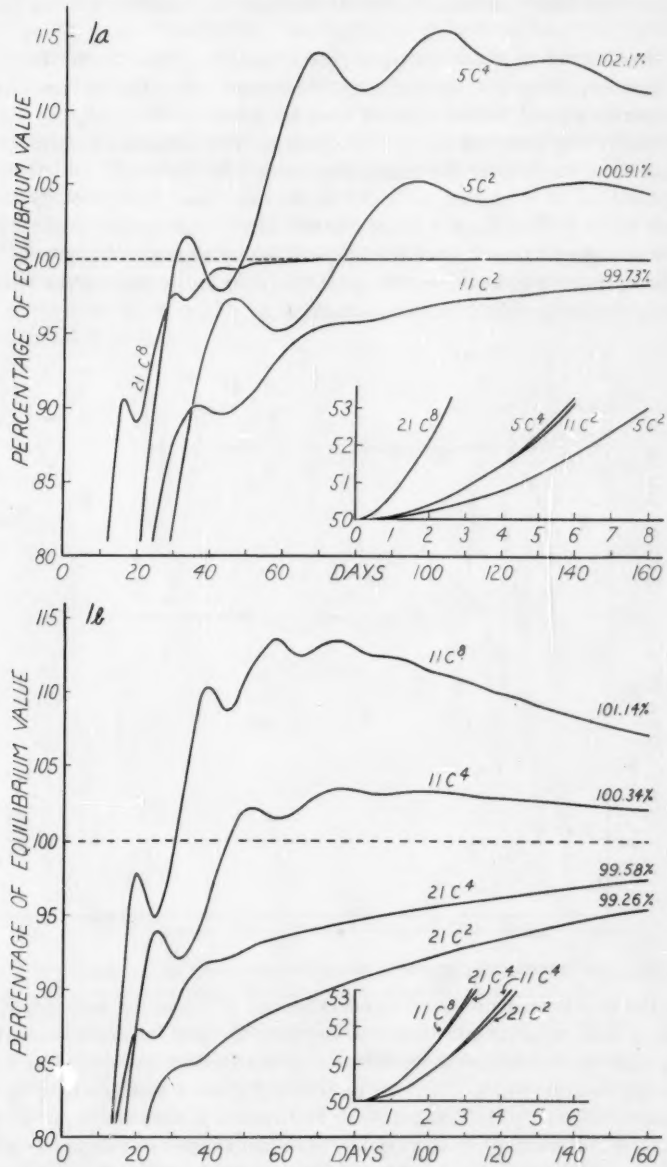
For antitemplate production by G, restoration of the total mass (M) was found to take place in spurts, with very little or no overshooting of the original size.[4] Both G and the antitemplate concentration (C) overshoot in all cases but both stabilize relatively rapidly, undergoing damped oscillations about their terminal equilibrium values.

If antitemplates are produced by D, the original size is reached more quickly and in a single spurt. An appreciable overshooting of considerable duration follows. G also overshoots but instead of stabilizing rapidly, it is sapped by continuing differentiation without being replenished commensurately by reproduction. As a result, G drops to a very low level before recovering. Even in the most favorable case investigated (antitemplate half-life of $3^1/_4$ days), G is sapped to a value far too low in relation to D to be biologically feasible.

A direct decision between these alternatives for antitemplate production could not be arrived at upon the basis of the limited manual calculations. Although G was sapped excessively for the case $D \rightarrow I$, it remained possible that it would become stabilized at higher values of k_5 and/or x. On the other hand, the system is quite stable for the alternative $G \rightarrow I$. However, the predicted early growth responses are too sluggish in all cases. This discrepancy between prediction and observation could be due simply to insufficient sensitivity of the model for the sample solutions which happened to be tested. Sensitivity can be increased by introducing higher rate of catabolic decay of antitemplates or by raising the number prerequisite for template inactivation to a value greater than two. In that case, the negative feedback would be proportional to a correspondingly higher power of the antitemplate concentration than the square. Neither of these adjustments is contraindicated by our present knowledge.

Exploration of the Model Using the IBM 709 Computer.—The first IBM 709 solutions were carried out to 365 days of compensatory growth of an adult organ after 50 per cent reduction in mass, using $^1/_{24}$-day integration intervals.

In this program, we investigated all combinations of antitemplate catabolic loss rates of $5^1/_3$ per cent, $10^2/_3$ per cent, and $21^1/_3$ per cent per day with inhibition proportional to C^2, C^4, and C^8 for both $G \rightarrow I$ and $D \rightarrow I$. The results closely confirm the accuracy of manual calculations (in so far as they overlap). However, G does not become stabilized for values of x greater than two ($I \alpha C^4$ or C^8) if antitemplates are formed by differentiated mass, as we had considered possible. Instead, the



Figures represent predictions of the model for compensatory adult organ growth after mass or antitemplate depletion for various parameter combinations. The percentages accompanying the curves at the far right designate the percentage of terminal equilibrium value attained after 365 days. The abbreviations yC^z accompanying the curves signify various parameter combinations as explained in note 5.

FIGS. 1*a*-1*b*.—Compensatory growth of total organ mass after 50% organ removal. The inserts in the lower right-hand corners are enlarged representations of the early growth.

system becomes highly unstable. Nor is the system stabilized for higher values of k_5.

Thus, the alternative of antitemplate production by D must be ruled out. Considering the various combinations of parameters tried, either the system is unstable, G is too greatly sapped, or the response is on the whole too sluggish.

Accordingly, the continuation of the program was limited to cases involving antitemplate production by the generative mass. In Figures 1a and 1b, compensatory growth of M is plotted for eight of the nine cases first investigated. The ninth case ($k_5 = 0.0533$, $I \alpha C^8$) is not plotted, for G is excessively sapped (to 73% for a corresponding value of D of 102.8%).[5] The progression of curves $5C^2$ to $5C^4$ to $5C^8$ shows that for k_5 as low as $5^1/_3$ per cent, four antitemplates per template is close to the upper limit for biological consistency.

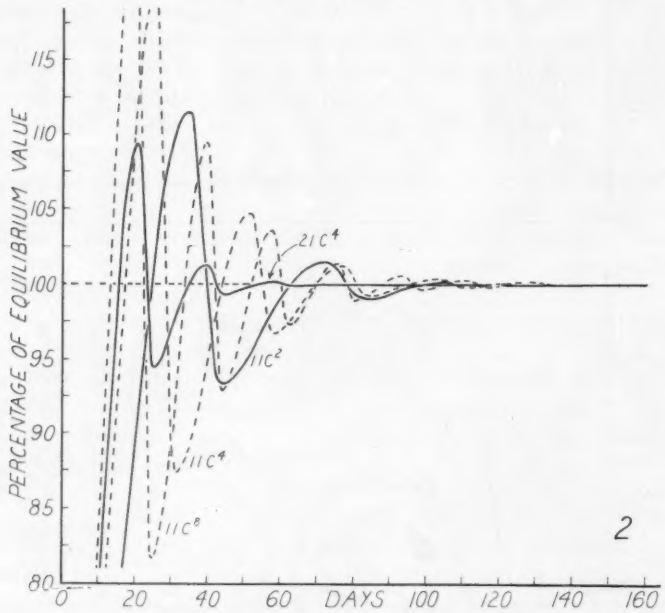


FIG. 2.—Compensatory growth of generative mass after 50% organ removal.

From the predicted kinetics of regeneration of M (Figs. 1a and 1b), it can be seen that a wide range is encompassed, bridging the gap between extensive overshooting and decided failure to overshoot. For a further assessment of biological consistency, the curves for G and C are plotted in Figures 2 and 3 for four representative cases: $11C^2$, $11C^4$, $11C^8$, and $21C^4$. The system is very stable for these cases (and for those not plotted), both variables stabilizing rapidly in damped oscillations about their equilibrium values. The maximum overshooting by C is 5.6 per cent.

Thus, for antitemplate production by G, the model defines a stable system which undergoes biologically plausible compensatory organ growth after 50 per cent mass reduction. This analysis does not, however, insure biological consistency and stability under other conditions of compensatory growth. It is possible, for ex-

ample, that a model system showing stable compensatory growth after 50 per cent mass reduction might be unstable after a greater or lesser reduction. Additionally, from the point of view of experimental confirmation, it is desirable to ascertain whether the model predicts compensatory overshooting or failure to overshoot for all ranges of mass reduction or only for certain ranges. Finally, it would be instructive to press the early growth rate to its upper limit by an appropriate selection of parameters. This is desirable because high early-growth rates have been observed experimentally.

In seeking the latter objective, a rate of antitemplate loss of 32 per cent (half-life of $2^1/_6$ days) and a template inactivation requiring 16 antitemplates have been selected as upper limits. An inhibitory reaction of this order cannot be ruled out (many combining sites might be present on the template molecule). Nor is a k_5 value of 0.32 implausible.

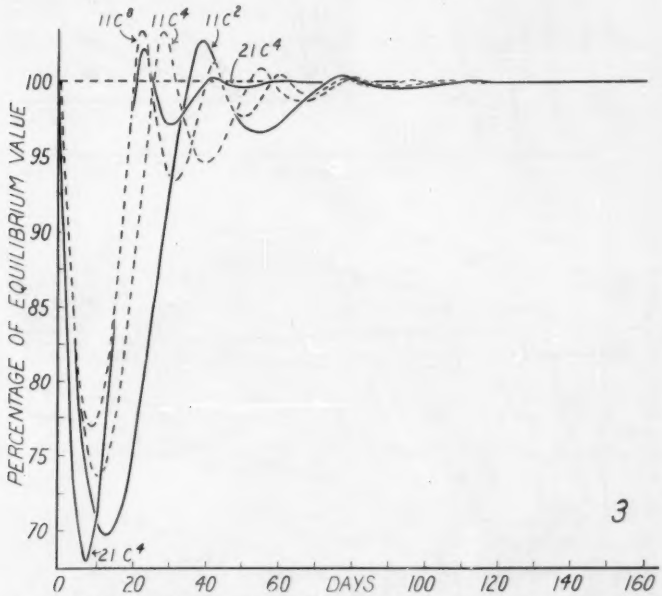
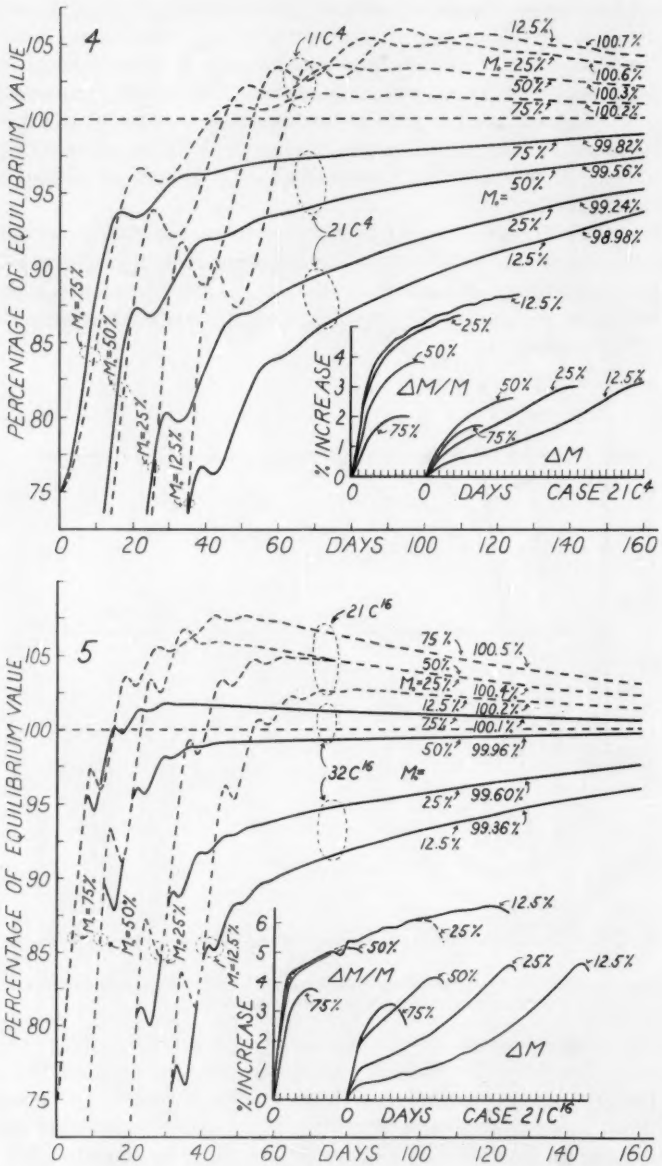


FIG. 3.—Changes in antitemplate concentration accompanying compensatory organ growth after 50 per cent organ removal.

For further assessment of the model, four cases ($11C^4$, $21C^4$, $21C^{16}$, and $32C^{16}$) were selected for extensive analysis. Predicted compensatory growth was determined for adult organ mass reduction of 25 per cent, 50 per cent, 75 per cent, and $87^1/_2$ per cent. The growth spurt after 10 per cent reduction in antitemplate concentration was also determined. The results are plotted in Figures 4 to 6. Additionally, in the lower right-hand areas of Figures 4 and 5, the absolute and relative changes in M are plotted for cases $21C^4$ and $21C^{16}$, respectively.

For case $11C^4$ (Fig. 4), M is found to overshoot in all instances. It does so the sooner the smaller the mass deficit, and to a greater degree, the larger the mass deficit. Although the initial growth spurt is more rapid, M does not overshoot for case



FIGS. 4-5.—Compensatory growth of total organ mass after organ depletion to residual masses of 12.5%, 25%, 50%, and 75% for four parameter combinations. The inserts in the lower right-hand corners show the early changes in mass, ΔM and $\Delta M/M$ for two of the four cases. ΔM is expressed as the percentage of the equilibrium mass and $\Delta M/M$ as the relative percentage increment.

$21C^4$ (Fig. 4), and equilibrium is attained asymptotically from below. This occurs sooner for lesser mass deficits but relatively slowly in all instances. As in case $11C^4$, M overshoots in all instances for case $21C^{16}$ (Fig. 5), the sooner the lesser the mass deficit. Case $21C^{16}$ differs from case $11C^4$, however, in that the extent of overshooting is greater the *lesser* the mass deficit (for the range investigated). Case $32C^{16}$ (Fig. 5) differs from the other three cases in several respects. Overshooting occurs for an initial mass of 75 per cent but fails to take place at lower initial masses. Nor does the approach to equilibrium take place in a monotonic sequence, there being a reversal between an initial mass of 50 per cent and 75 per cent. Thus, the asymptotic approach to equilibrium from above in the 75 per cent case is not more rapid than that from below in the 50 per cent case. Otherwise, the same progression of rate of approach to equilibrium holds for the case $32C^{16}$ as for case $21C^4$. The initial spurt of growth is more rapid for equivalent mass deficits in case $32C^{16}$ than in case $21C^{16}$.

A feature common to all of the compensatory growth curves for total mass (for $G \rightarrow I$) is their undulatory character. This is caused by the characteristic damped

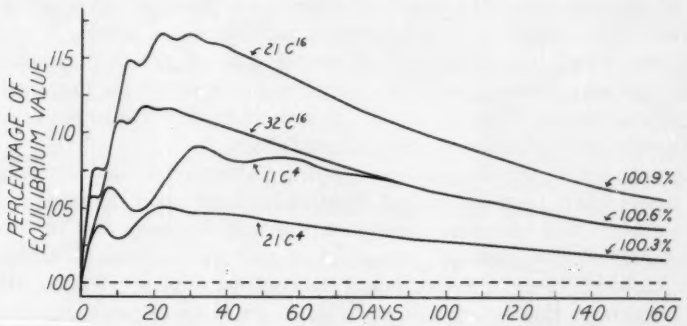


FIG. 6.—Growth spurt of the total mass after 10% reduction in antitemplate concentration.

oscillatory approach of the generative mass to equilibrium as it successively overshoots and undershoots its equilibrium value (Fig. 2). The effects on M resulting therefrom are both direct and indirect. The first undulation of M is always the greatest. It almost always occurs before the first overshooting, resulting in a marked although temporary deceleration of growth rate which usually leads to temporary reduction in mass. This course of compensatory growth is different from that predicted in the event of antitemplate production by differentiated mass, where short term undulations or decelerations do not occur (see Fig. 10 of reference 2). As is noted below, this distinctive undulatory behavior provides a basis for discounting the alternative $D \rightarrow I$ independently of that considered above. The experimental data, although meager, bear directly on this matter.

Reference to Figure 6 revea's that an appreciable spurt of growth could be engendered in a relatively short time by a 10 per cent reduction in antitemplate concentration. Seen clearly is the fact that increasing the value of x leads to a greater excess of growth, whereas increasing the value of k_5 leads to a reduction in excess growth.

Discussion.—Antitemplate source: It was suggested earlier[2] that for $G \rightarrow I$, the size of an organ system would be less critically determined than it would be for $D \rightarrow I$. This suggestion was based on the fact that in the former case, the feedback stems ultimately from the generative mass, which comprises a minor percentage (7.15%) of the total mass, whereas in the latter case the direct regulatory function is assigned to the bulkier of the two components. It is now clear, however, that if inhibitory control is assigned to D, the overshooting of D inhibits the growth of G for such an extended period that G is drained to unreasonably low levels.

In the derivation of the model,[2] two other observations also pointed to G rather than to D as the antitemplate source. First, the growth curve for G (for normal chicken growth) more nearly resembled that for the feedback (Fig. 2 of reference 2) than did the curve for D. Second, it was noted that for the case $D \rightarrow I$, there would be a period during normal development when the relative volume of the dilution pool would dip slightly below its terminal value, which is an unlikely occurrence.

A cogent and gratifying experimental confirmation of G rather than D as the source of antitemplates is the finding by Higgins and Anderson[6] and Brues, Drury, and Brues[7] that, in partially hepatectomized rats, there is a slowing down of liver growth after 6 to 7 days followed by a second spurt of growth. Although these investigators were unable to offer any convincing explanation for this observation, it is readily accounted for by our model. It reflects the first and largest undulation, caused by the first oscillation of G, as discussed above.

At this point it is perhaps appropriate to direct attention to the inner consistency of the model where functions of and negative feedback upon the generative mass are concerned. Our major assumption in this area has been that of a negative feedback control of normal and compensatory growth mediated by a progressive specific inhibition of the reproduction of the generative mass. When applied, the model based upon this assumption led to disclosure of the necessity of also placing *differentiation* of G under negative feedback control. Upon reflection, however, this second requirement would seem to follow logically from the first. If the one function of G is controlled by a negative feedback involving specific combination of antitemplates with templates, i.e., by a steric clogging of the machinery for reproduction, it would seem reasonable to expect this steric clogging to also interfere with the other function. Thus, the dictate referred to above appears to be logically consistent with one of the major premises upon which the model is based.

Subsequent application of the model to compensatory organ growth dictated the further necessity of G itself being the production source of the antitemplates. This result could not have been asserted *a priori* to be inevitable. However, it is fully consistent with, and would also appear to follow logically from, our assumptions, particularly that of a *specific* inactivation of templates by complementary particles. The reasons for this are (*a*) that the most likely production source of particles complementary to templates or portions thereof is the templates themselves and (*b*) that the templates are the more dynamic components of the organ system, from which all its mass ultimately derives and from which new mass arises continually.

These required elaborations of our model, being logical and reasonable, strengthen our confidence in the validity of the basic premises upon which it is founded.

Rate of loss of antitemplates; number of antitemplates required for template inactiva-

tion: The foregoing parameters determine sensitivity to departures from equilibrium. They are magnifying or reducing factors, which, in being varied, increase or decrease this sensitivity. Our calculations abundantly demonstrate that an increase in either k_5 or x leads to greater initial sensitivity, i.e., to greater early growth rates. In the subsequent response, however, the effects of these two factors are opposed; higher x values tend to promote overshooting, and higher k_5 values tend to prevent or reduce overshooting. In practice, a daily percentage catabolic loss of antitemplates of from $1^1/_4$ to 5 times the order of the inhibitory reaction leads to a reasonable balance between the two factors. Reaction orders of from 2 to 16 and catabolic rates of from $5^1/_3$ to 32 per cent per day can, in appropriate combinations, lead to biologically resonable compensatory organ growth curves. However, from the combined point of view of rapidity of early response and time of first attainment of equilibrium value, a reaction order of 4 would appear to be near the lower limit, in so far as the meager experimental data at hand are concerned.

In general, rapid restoration of stable equilibrium for systems stabilized by negative feedback apparently demands overshooting to some extent, at least, as a necessary condition. It is worth noting that our model is not exceptional in this regard; although restabilization of total mass can occur without overshooting, overshooting of G and C, though rapidly damped, is always involved.

Nature of the antitemplates: Little is known about the identity of the antitemplates. According to the final version of the model, their production occurs at a rate proportional to the amount of generative mass present ($G \rightarrow I$). These kinetics suggest that antitemplates are either products or by-products of the functioning and/or maintenance of G or of some component thereof. Their formation solely in the process of reproduction or differentiation of G has, however, been ruled out.

In the case of the regenerating liver, Glinos' studies [8–10] indicate that control is exerted by plasma proteins, synthesis of which occurs largely in the liver.[11] The reader is referred to the literature[8, 11, 12] for studies pertaining to the marked changes in plasma proteins accompanying partial hepatectomy (and even laparotomy alone). It suffices to say that plasma protein levels become markedly and rapidly depressed. In some cases, maximal depression occurs within 24 hours. Recovery times vary from 3 to 28 days, depending on the fraction. We note in this connection that the predicted drop in antitemplate concentration (Fig. 3) is extremely rapid following partial organ removal. The relatively rapid recovery involves a quick damping about the equilibrium level, being very close to equilibrium by the 32nd day, at the latest.

In this connection, Greenbaum, Greenwood, and Harkness[13] estimated that plasma protein synthesis in the regenerating rat liver reaches its maximum rate considerably (24 to 60 hours) later than does liver cell protein synthesis (assuming a plasma protein half-life of 3 to 4 days). They therefore concluded that the two processes are independent, for they appear to behave as unrelated systems, i.e., they concluded that the plasma protein level could not control liver regeneration. In addition to the arguments presented by Glinos[8] against this view, we can add one provided by our model. Analysis of the predictions of the model shows that it is characteristic of this system that the maximum increase in total organ mass *precedes* the maximum increase in antitemplate concentration (by from 1 to 7 days

for those cases investigated). For a mass deficit of 75% the lag ranges from 2 to 3 days. This finding provides an additional instance in which a prediction of the model accords with conclusions based upon experiment. Contrary to the assumption of Greenbaum, Greenwood, and Harkness, it follows that the time lag between attainment of maximum rates of increase of liver cell protein and plasma protein does not preclude a direct link between these two processes.

Correlations with experimental findings: Unfortunately, experimental work on compensatory organ growth has not been designed along appropriate lines nor sufficiently refined to answer most of the questions upon which we have focused attention. Additionally, it has been carried out almost entirely with immature rats and mice, whereas our model has been quantified for the chicken and its predictions derived primarily for the adult.[14] Certain useful comparisons and correlations can nonetheless be drawn.

Compensatory growth of the young rat kidney has been studied by Rollason[15] and others. In unilaterally nephrectomized 14-day old animals, the remaining kidney attained 90 per cent of the combined weight of two control kidneys (relative to body surface) after 19 days. Maximum enlargement occurred shortly after 20 days. Results vary considerably between different investigators and animals of different ages (see ref.[15]). Disregarding for the moment the fact that these findings are for immature rats rather than mature chickens, we can assert that, in so far as they go, they are not greatly out of line with some of our curves for compensatory growth after 50 per cent mass reduction.

The greatest amount of compensatory growth study has been devoted to the rat liver. From these studies many interesting points emerge. Greenbaum, Greenwood, and Harkness[13] have computed an average curve based upon literature values for compensatory liver growth after 66–70 per cent hepatectomy in immature rats. The results average out to mass restorations of about 20, 40, 50, and 58 per cent after 1, 2, 3, and 4 days. Our predictions are far out of line with these results. The greatest early mass restorations predicted are for the case $32C^{16}$. For a 50 to 75 per cent deficit, the restoration amounts to only about 0.79 to 1.34 per cent and 4.46 to 8.48 per cent of the adult mass after the first and fourth days respectively.

Aside from that of species difference, the first explanation that comes to mind for this discrepancy is that the rate of organ mass restoration may be higher in the immature animal than in the mature one—to which our predictions apply. This has indeed been found to be the case in rats[16, 17] and mice,[18] in which a definite decrease in the rate of regeneration of liver mass is found with increasing age. We can also appeal to the model for an answer to the question of change, if any, in compensatory regenerative capacity with age. However, only very limited calculations have been carried out thus far for regeneration in the immature animal. Compensatory growth in the 40-day old chick was investigated[2] for the case $10C^2$ for mass deficits of 50 and 75 per cent. M was found to overshoot the standard mass for the corresponding age (i.e., the control mass) after about 39 and 48 days respectively (Fig. 12 of reference 2). The corresponding masses after the same interval in the adult are only 91 and 84 per cent of the equilibrium mass, and overshooting fails to occur.

Although these findings help to reconcile the discrepancy, they could not possibly

suffice as the explanation, for the rate of compensatory liver growth is also relatively high in adult rats.[16] A factor of possibly greater importance is the fact that liver regeneration appears to involve ancillary processes of mass increase superimposed upon the basic compensatory growth process. Our model, of course, does not take ancillary processes into account in its present form. Thus, in rat and mouse liver regeneration, it has been shown by many workers (see Yokoyama *et al.*[18] and Harkness[19] for references) that much of the material added to the liver in the great mass increase of the first two days represents neutral fat. This neutral fat (and certain other substances) is rapidly mobilized into liver parenchymal cells with a probable heavy drain on body reserves.[18] Moreover, no mitoses occur in the first 24 hours. In view of these findings, the liver would appear to be an unfavorable organ for studying the major carrier wave of growth and growth control which our model is intended to represent.

Concerning evidence for overshooting, experimental methods have not as yet been sufficiently refined to demonstrate unequivocally that it does or does not occur. Some scattered data appear to show the effect,[6, 16] although it has been largely overlooked or discounted as due to experimental error.

Future and Present Value of the Model.—By reason of the explicitness of the model, several possible mechanisms considered in our general scheme of growth control have been ruled out as unrealistic. Of the various alternative sources for the antitemplates, several independent considerations have focused attention on their continual production by generative mass as the only one reasonable. The model adequately reproduces a number of phenomena observed in the compensatory response to disturbance of equilibrium of organ systems, as well as reproduces their normal growth. Thus, it reproduces the spurt of compensatory growth of an organ system following artificial reduction either at equilibrium or during the growth phase. It predicts spontaneous resumption of organ growth after artificial reduction of antitemplate concentration at terminal equilibrium, observed for liver by Glinos[8] in plasmapheresis experiments with adult rats. It predicts the secondary spurt of growth observed to occur after the first week or so in partially hepatectomized rats.[6, 7] It anticipates that liver cell protein synthesis in the regenerating rat liver will attain its maximum rate before plasma protein synthesis does,[13] and it predicts the decrease in the rate of regeneration of organ mass with increasing age.

Additionally, the model directs attention to a number of questions to be decided by future experiments to elucidate the mechanisms of growth control. For example, after partial organ removal,

1. Does overshooting of total organ mass occur and, if so, to what extent?

2. If overshooting occurs, does it occur over the entire range of experimentally produced mass deficits?

3. If overshooting occurs, but not over the entire range, does it fail to occur at greater or at lesser mass deficits?

4. If overshooting occurs, does its magnitude increase or decrease with increasing mass deficit?

5. If overshooting occurs, does it occur the later the greater the mass deficit or the lesser the mass deficit?

6. If there is failure to overshoot, does attainment of equilibrium occur more rapidly for lesser mass deficits?

7. Do the compensatory growth curves for total organ mass show short-term undulations in their attainment of equilibrium?

8. If undulations and overshooting occur, does the first undulation occur before or after the first overshooting?

9. If undulations occur, is there actual mass regression in decelerated growth phases, etc.?

When further experimental information on these points is available, we will be able to take a long step forward in deciding the relevancy of our model and of its several solutions (and of the various parameter combinations upon which they are based).

Summary.—The mathematical model for the major carrier wave of growth and growth control proposed by Weiss and Kavanau[2] has been tested for the case of compensatory organ growth in the adult by employing an IBM 709 Digital Computer. Upon the basis of these tests it must be concluded that the antitemplates are produced by the generative mass and that four or more of them are required to inactivate each template. Several sample solutions have been determined; in all the cases, the compensatory growth curves for total mass are undulatory in character. This finding is consistent with observations on liver growth following partial hepatectomy in the rat. The model also reproduces the spurt of compensatory growth of an organ system following artificial mass reduction, predicts spontaneous resumption of organ growth after artificial reduction of antitemplate concentration at terminal equilibrium, anticipates that liver cell protein synthesis in the regenerating rat liver will attain its maximum rate before plasma protein synthesis does, and predicts the decrease in rate of regeneration of organ mass with increasing age. Additionally, the model directs attention to a number of questions to be decided by future experimentation.

* Supported in part by Research Grant No. G-5663 from the National Science Foundation.

† The terms and symbols used in this paper are as follows:

b	ratio between actual feedback inhibition at terminal equilibrium and potential complete inhibition
C	concentration of antitemplates
D	differentiated mass of the organ (or organ system)
$D \rightarrow I$	antitemplates produced continually by D
e	subscript referring to terminal size
$I \alpha C^x$	feedback inhibition proportional to the xth power of C
G	generative mass of the organ (or organ system)
$G \rightarrow I$	antitemplates produced continually by G
I	number of antitemplate molecules
k_1	rate constant for the accretion component of differentiation
k_2	rate constant for the maintenance component of differentiation
k_3	rate constant for the catabolic loss of D
k_4	rate constant for catabolic loss of antitemplates
log	natural logarithm
M	total mass of the organ (or organ system)
V	volume of the dilution pool
x	number of antitemplate molecules required to inactivate one template

[1] Weiss, P., *Yale J. Biol. and Med.*, **19**, 235–278, 1947.

[2] Weiss, P., and J. L. Kavanau, *J. Gen. Physiol.*, **41**, 1–47, 1957.

[5] These calculations would have required many thousands of years to carry out by the previously used manual procedure.

[4] Failure to overshoot the original size, for the case $G \rightarrow I$, eventually proved to be purely accidental, a consequence of the particular parameter combinations selected for testing.

[5] Hereafter and in the figures, the abbreviation yC^x will be used, y designating the daily percentage catabolic loss of antitemplates (rounded off to 5, 11, 21, or 32) and x the number of antitemplates required for the inactivation of one template.

[6] Higgins, G. M., and R. M. Anderson, *Arch. Path.*, **12**, 186–202, 1931.

[7] Brues, A. M., D. R. Drury, and M. C. Brues, *Arch. Path.*, **22**, 658–673, 1936.

[8] Glinos, A. D., in *The Chemical Basis of Development* (Baltimore: The Johns Hopkins Press, 1958), pp. 813–839.

[9] Glinos, A. D., *Science*, **123**, 673–674, 1956.

[10] Glinos, A. D., *Fed. Proc.*, **15**, 76, 1956.

[11] Roberts, S., and A. White, *J. Biol. Chem.*, **180**, 505–516, 1949.

[12] Chanutin, A., J. C. Hortenstine, W. S. Cole, and S. Ludewig, *J. Biol. Chem.*, **123**, 247–256, 1938.

[13] Greenbaum, A. L., F. C. Greenwood, and E. D. Harkness, *J. Physiol.*, **125**, 251–262, 1954.

[14] Predictions for compensatory organ growth in the immature animal are also within the scope of the model. These involve more complex computations and have not yet been programmed for the IBM 709, although they have been explored manually.[2]

[15] Rollason, H. D., *Anat. Record*, **104**, 263–285, 1949.

[16] Bucher, N. L. R., and A. D. Glinos, *Cancer Res.*, **10**, 324–332, 1950.

[17] Norris, J. L., J. Blanchard, and C. Povolny, *Arch. Path.*, **34**, 208–217, 1942.

[18] Yokoyama, H. O., M. E. Wilson, K. K. Tsuboi, and R. E. Stowell, *Cancer Res.*, **13**, 80–85 1953.

[19] Harkness, R. D., *Brit. Med. Bull.*, **13**, 87–93, 1957.

ERRATUM

In Dr. Harmon Craig's paper entitled "The Thermodynamics of Sea Water," appearing in the September issue of this volume of these PROCEEDINGS, the sentence beginning on line 18 of page 1223 should read as follows:

"This is equivalent to partial partial Gibbs free energy, a term which has no meaningful defininition."

INFORMATION TO CONTRIBUTORS

THE PROCEEDINGS is the official organ of the NATIONAL ACADEMY OF SCIENCES and of the NATIONAL RESEARCH COUNCIL for the publication of brief accounts of important current researches of members of the ACADEMY and of the COUNCIL and of other American investigators. The PROCEEDINGS will aim especially to secure prompt publication of original announcements of discoveries and wide circulation of the results of American research among investigators in other countries and in all branches of science.

ARTICLES should be brief. The viewpoint should be comprehensive in giving the relation of the paper to previous publications of the author or of others and in exhibiting, where practicable, the significance of the work for other branches of science. Elaborate technical details of the work and long tables of data should be avoided, but authors should be precise in making clear the new results and should give some record of the methods and data upon which they are based.

MANUSCRIPTS should be prepared with a current number of the PROCEEDINGS as a model in matters of form, and should be typewritten in duplicate with double spacing, the author retaining one copy. Illustrations should be confined to text figures of simple character, though more elaborate illustration may be allowed in special instances to authors willing to pay for their preparation and insertion. Particular attention should be given to arranging tabular matter in a simple and concise manner.

MANUSCRIPTS will be accepted only from members of the Academy who will assume responsibility for the propriety and scientific standards of the paper and for any printing costs in excess of those allowed them by the Academy. Manuscripts should be addressed to THE PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, 2101 Constitution Ave., Washington 25, D. C.

GALLEY PROOF will be sent, with the understanding that charges for author's corrections shall be billed to him. Authors should therefore make final revisions on the typewritten manuscripts. Original artwork or figures will be returned if requested when the manuscript is submitted. Because of time limitations, page proofs will not be sent.

REPRINTS should be ordered when proof is returned, on the reprint order form which will be enclosed with the proof. They will be furnished at cost, approximately as follows:

SCHEDULE OF REPRINT RATES (POSTAGE NOT INCLUDED)

Quantity	up to 4 pp.	up to 8 pp.	up to 12 pp.	Standard Covers
50	$ 7.85	$15.10	$22.30	$6.90
100	8.95	17.30	25.60	8.40
150	10.10	19.50	28.95	9.95
add'l 100's	2.20	4.40	6.60	3.05

CONTENTS

Page

ANATOMY.—THE LOSS OF PHENOTYPIC TRAITS BY DIFFERENTIATED CELLS IN VITRO, I. DEDIFFERENTIATION OF CARTILAGE CELLS . . . . . *By H. Holtzer, J. Abbott, J. Lash, and S. Holtzer* 1533

BIOCHEMISTRY.—SOME OBSERVATIONS ON THE SYNTHESIS AND FUNCTION OF THE PHOTOSYNTHETIC APPARATUS IN *Rhodospirillum rubrum* . . . . . *By Germaine Cohen-Bazire and Riyo Kunisawa* 1543

BIOCHEMISTRY.—INTERACTION OF SOLUBLE RIBONUCLEIC ACID AND MICROSOMES . . . . . *By Mahlon B. Hoagland and Lucy T. Comly* 1554

BIOCHEMISTRY.—ANALYSIS OF SEQUENCE PATTERNS IN RIBONUCLEASE, I. SEQUENCE VECTORS AND VECTOR MAPS . . . . . *By Frank Lanni* 1563

BIOCHEMISTRY.—RECONSTITUTION OF THE DINITROPHENOL-SENSITIVE ATP-ADP EXCHANGE REACTION OF OXIDATIVE PHOSPHORYLATION . . . . . *By Charles L. Wadkins and Albert L. Lehninger* 1576

BIOCHEMISTRY.—A FACTOR WHICH INCREASES THE DINITROPHENOL-SENSITIVITY OF THE ATP-ADP EXCHANGE REACTION OF OXIDATIVE PHOSPHORYLATION . . . . . *By Charles L. Wadkins and Albert L. Lehninger* 1582

GENETICS.—ON THE MUTAGENIC EFFECT OF ALKYLATING AGENTS . . . . . *By Ekkehard Bautz and Ernst Freese* 1585

MATHEMATICS.—SOME SINGULAR MIXED PROBLEMS . . . . . *By Robert Carroll* 1594

MATHEMATICS.—OPTIMAL GAMBLING SYSTEMS . . . . . *By Lester E. Dubins and Leonard J. Savage* 1597

MATHEMATICS.—FIELDS OF UNIT VECTORS IN THE FOUR-SPACE OF GENERAL RELATIVITY . . . . . *By Luther P. Eisenhart* 1598

MATHEMATICS.—GENERALIZED SPACES OF GENERAL RELATIVITY, II . . . . . *By Luther P. Eisenhart* 1602

MATHEMATICS.—SPACES WHICH ADMIT FIELDS OF NORMAL NULL VECTORS . . . . . *By Luther P. Eisenhart* 1605

MATHEMATICS.—A METHOD FOR DEFINING PRINCIPAL MODES OF NONLINEAR SYSTEMS UTILIZING INFINITE DETERMINANTS . . . . . *By Demetrios G. Magiros* 1608

MATHEMATICS.—INVESTIGATIONS ON FINITE GROUPS . . . . . *By Michio Suzuki* 1611

MATHEMATICS.—CONVERGENCE IN NORM . . . . . *By G. T. Whyburn* 1614

MICROBIOLOGY.—SIGNIFICANCE OF CONTINUED VIRUS PRODUCTION IN TISSUE CULTURES RENDERED NEOPLASTIC BY POLYOMA VIRUS. . . . . *By Renato Dulbecco and Marguerite Vogt* 1617

MICROBIOLOGY.—THE SPARING EFFECT OF RMC POLIOVIRUS ON PRIMARY AMNION CELL CULTURES . . . . . *By Monto Ho* 1623

MICROBIOLOGY.—REGULATION BY COLIPHAGE LAMBDA OF THE EXPRESSION OF THE CAPACITY TO SYNTHESIZE A SEQUENCE OF HOST ENZYMES . . . . . *By Michael B. Yarmolinsky and Herbert Wiesmeyer* 1626

PHYSICS.—INVARIANT IMBEDDING AND THE REDUCTION OF TWO-POINT BOUNDARY VALUE PROBLEMS TO INITIAL VALUE PROBLEMS . . . . . *By Richard Bellman, Robert Kalaba, and G. Milton Wing* 1646

PHYSICS.—TESTS OF FORM OF THE ONE-PION EXCHANGE POTENTIAL . . . . . *By G. Breit, M. H. Hull, Jr., K. E. Lassila, and H. M. Ruppel* 1649

PHYSIOLOGY.—A MODEL OF GROWTH AND GROWTH CONTROL IN MATHEMATICAL TERMS, II. COMPENSATORY ORGAN GROWTH IN THE ADULT . . . . . *By J. Lee Kavanau* 1658

ERRATUM.—THE THERMODYNAMICS OF SEA WATER . . . . . *By Harmon Craig* 1673